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13. ABSTRACT ***(Maximum 200 Words)***
We proposed to establish an approach by which tumor cells are eradicated through selective induction of $CD8^+$ T cells specific for a protein overexpressed in many adenocarcinomas. A peptide derived from *HER-2/neu* (HN654-662, GP2) stimulates cytotoxic T lymphocytes (CTL) that lyse primary tumors from ovarian or breast cancers. It has been proposed that the poor immunogenicity of GP2 is due to poor binding to HLA-A2.1. Our data demonstrate GP2 is an extremely poor HLA-A2.1 binding peptide. Furthermore, modifications to anchor residues, predicted to improve binding, do not significantly increase affinity. We have determined the structure of A2/GP2 and show that the center of the peptide is disordered. Combinations of anchor substitutions and another substitution (V5L) that had improved affinity show reduced affinity compared to the individual substitutions. This indicates that there are significant interactions between the different residues bound to HLA-A2.1. Individual substitutions at positions 3, 5, 6 and 7 do not significantly increase affinity. Transgenic $A2K^b$ mice do not generate a response to GP2 either as peptide or bound to dendritic cells. Some of the variants of GP2 do generate a CTL response, but do not recognize GP2-pulsed targets well. Mice doubly transgenic for $A2K^b$ and HER-2/neu (MMTV promoter) generate spontaneous tumors around 5 months of age. Peptide immunization can induce CTL responses towards HER-2/neu peptides presented by the tumor.

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5. Introduction

Cytotoxic T lymphocyte (CTL) responses to tumors in both man and animal models have been demonstrated [1-4]. Tumor cells are autologous and should be immunologically ignored, but many are recognized. These tumor cells may express mutated proteins, new proteins encoded by transforming viruses, or proteins normally expressed at low levels, but now are expressed at high levels. The primary discriminator of T cell activation is the interaction of the T cell receptor (TcR) and the MHC molecule. It is this interaction that the goals of this proposal seek to enhance.

Class I MHC molecules are ternary complexes found on the plasma membrane of nearly all cells in the body. These molecules contain a polymorphic heavy chain, β_2-microglobulin (β_2m) and a small peptide (typically 8-10 amino acids). The heavy chains are synthesized, co-translationally translocated into the endoplasmic reticulum (ER), associate with the molecular chaperones calnexin and calreticulin and with peptide-loading molecules such as p48 and TAP [5]. Only complexes that are completely assembled with peptide and β_2m are allowed to egress from the ER into the Golgi [6]. The availability of peptide appears to be the rate-limiting step in cell surface expression of class I proteins [7].

An analysis of peptides that bind to specific class I molecules show some positions in the peptide that are relatively invariant [8, 9]. These amino acids interact with substructures of the MHC molecule called pockets [10]. Originally the anchor side chains were believed to provide the majority of the free energy of binding. Subsequent analyses by our laboratories and others have clearly demonstrated that a peptide's binding ability depends on positive and negative effects from all residues within the peptide [11-13]. Possession of amino acid side chains, which would be favorable anchors, is not sufficient to make a peptide bind; nor is the absence of the residues sufficient to render a peptide unbindable. Indeed, many peptides, which seem as likely to fit, do not function as epitopes to T cells *in vivo* [14] suggesting that they do not bind to the class I molecules.

The issue of peptides binding to class I molecules is important since peptides which are <u>not</u> bound to class I and are <u>not</u> present on the surface, are <u>not</u> immunogenic. However, the exact effect of the affinity of peptide for class I, the stability of the complex on the cell surface and its subsequent immunogenicity is a matter of conjecture at the present time.

This interaction between heavy chain and peptide is crucial not only in the generation of CTL responses, but also in thymic selection. Class I MHC/peptide complexes are required for the egress of mature T cells from the thymus and are important in both positive and negative selection during T cell development [15, 16]. Recent experiments have shown that during thymic education the fate of the T cell (deletion or proliferation) is dependent on the affinity of the class I MHC/peptide complex and the T cell receptor [17]. Therefore, the affinity between TCR and class I MHC/peptide complex is critical for function.

A *HER2/neu* derived peptide has been identified that is recognized by autologous CTL [18]. This peptide HN654-662 (IISAVVGIL), has the HLA-A2.1 binding motif [8] and has been shown to stimulate CTL from tumor infiltrating lymphocytes derived from breast and ovarian tumors [19]. However, CTLs stimulated by HN654-662 exhibit poor cytotoxicity possibly due to the peptides poor solubility and poor binding affinity. To gain further insight into the factors that govern CTL activity, we examined the binding of HN654-662 to recombinant HLA-A2.1. As seen in our preliminary results, this peptide is extremely unusual for a peptide that stimulates

CTL activity. HN654-662 marginally binds HLA-A2.1 and modifications shown to increase the affinity of other peptides ([20] and unpublished data) have little effect. Therefore, this peptide gives us the rare opportunity to use structural biology as a tool to solve an important biological problem in a timely fashion. The crystallographic structure of HN654-662 will provide information to explain the poor binding of the peptide.

Structural biology gives insights into function/importance that are not apparent from other data. For example, groups have identified peptides that do not appear to bind to class I MHC molecules in the usual manner [21]. It was the crystal structure of a HLA/peptide complex that demonstrated that the peptide extended out of the carboxyl terminus [22]. Recent advances in technology have increased the speed at which structures may be determined to the extent that structural biology is now a useful tool to probe function. For example, a class I histocompatibility complex structure can go from an idea to a finished structure solved by molecular replacement in 2-3 months. We believe our extensive experience in the biophysical studies of class I MHC/peptide interactions and class I MHC mediated CTL killing will allow us to enter a new field, cancer immunotherapy, and make significant contributions.

Experimental Methods (Derived From Statement of Work)

Specific Aim 1. Develop HN654-662 variant peptides with improved affinity for HLA-A2.1

A The first task is to determine the co-crystal structure of HLA-A2.1 complexed with wild-type HN654-662 peptide. Objective: Complete by 12/96.

Methods: A soluble recombinant form of HLA-A2 is folded in vitro in the presence of β2m and HN654-662. The protein is purified by gel filtration chromatography. It is concentrated to 10 mg/ml and buffer exchanged to 25 mM MES pH6.5 for crystallization trials. Initial crystals that form are crushed to make seed crystals for additional trails. Large single crystals are transferred to cryoprotectant and rapidly cool to -180°C by plunging into liquid propane. The crystals are stored as solid propane popsicles in liquid nitrogen until use. Crystallographic data are collected and the structure determined by molecular replacement methods.

Progress: We have finished the structure of HN654-662 bound to A2 it is published in The Journal of Biological Chemistry. A reprint is enclosed as Appendix I. The structure shows that the peptide does not bind well because the center of the peptide does not make stabilizing contacts with the MHC peptide-binding groove.

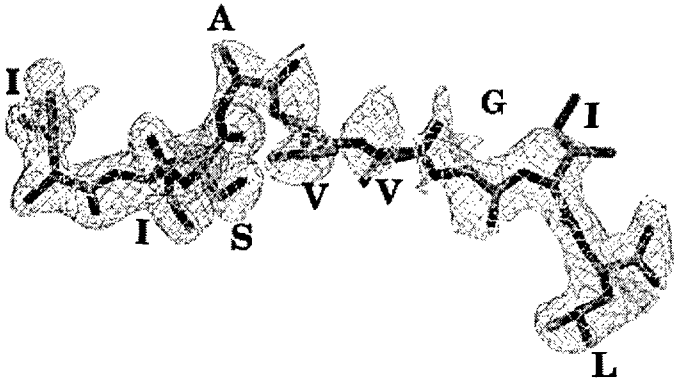


FIGURE 1. The center of the HN654-662 peptide is disordered. The averaged omit electron density map of the HN654-662 peptide with a cover radius of 1.5 Å. A2 has been removed for clarity. For more details see our manuscript attached as an appendix.

B Using the crystal structure, identify an amino acid that points down into the peptide binding cleft. Synthesize a peptide library with 20 different peptides. Fold HLA-A2 with library and isolate stabilizing peptides. Identify residue that points up towards TCR. Complete by 2/97

Methods: The library may be synthesized using standard FMOC chemistry on a solid phase synthesizer. The position to be randomized is coupled with a mixture of 19 amino acids (cysteine left out to reduce difficulties in the folding reaction). The difficulty with the library is that salts, etc that cannot be effectively purified away inhibit folding of our protein. However, the library may be added to the folding cocktail of A2 and protein isolated as described. Folded protein will contain those peptides that allow for productive complexes. The peptides may be isolated after treatment by spinning through a centricon-3 filter apparatus (Amicon) and identified by mass spectrometry.

Progress: In the absence of the crystal structure, we had produced one library randomizing position 3. Position 3 has been shown to be a secondary anchor in many peptides. As the peptide only has one polar residue and it is the serine at P3, we decided to substitute the first position from isoleucine to lysine to improve solubility of the peptide library. The library was used in our *in vitro* folding reaction and large quantities of A2 were isolated. The protein is not very stable however. Warming the mixture to room temperature resulted in complete denaturation of the complex. We repeated the experiment keeping the preparation cool at all times. The protein was sent to collaborators at Zycos Inc who are very talented mass spectroscopists. Based on the mass data, all of the 19 amino acids in the library were present bound to A2. Since mass spectrometry is not quantitative, it suggests that this technique is not viable for this goal. We intend to repeat the experiment and sequence the eluted peptides by Edman degradation. This will give us a quantitative measurement of the frequency of each amino acid at position 3. This has not been completed as of October 2000 because we have focused on the single amino acid substitutions and chimeric peptides described below.

With the crystal structure in hand, we have found that position five points mostly sideways (Figure 2). The remaining residues within the cleft are not well defined (as described above and in the conclusions) and we are unable to predict which residues should be pointing down and which up. A substitution of valine at position five with leucine results in a peptide with higher affinity (Figure 3). The residues that do not appear to be important for TcR recognition will be our next targets for library formation. We have not attempted this yet because we have been unable to design peptides that bind with high affinity (see C below).

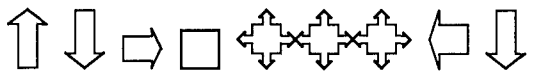


Figure 2. Orientations of the amino acids of H654-662 when bound to HLA-A2. Arrows pointing in all four directions: we cannot reliably position these amino acids; Up arrow: towards TcR; Down arrow: towards beta pleated sheet; Right arrow: towards α2 α helix; Left arrow: towards α1 α helix.

C Chemically synthesize peptides that improve stability and determine thermostability. Complete by 4/97.

Progress: We hypothesized that the central residues in HN654-662 were not conducive to forming stable interactions with the A2 binding cleft. Therefore, in order not to bias the study, we synthesized a variety of ligands with substitutions of HN654-662 at positions 5-7 and tested for binding affinity. We measured a relative binding constant by adding peptide exogenously to T2 cells that lack a functional peptide transporter. A2 on the surface of these cells are relatively peptide receptive and by virtue of the peptide binding, the A2 is stabilized on the cell surface. The amount of A2 on the surface can be followed with antibodies and the quantified by flow cytometry.

As can be seen from Table I, we were totally wrong in our hypothesis. Most of the substitutions did not improve affinity. The only one that did is V5L and we can explain that from the crystallographic structure.

Name	Sequence	K_r	Name	Sequence	K_r	Name	Sequence	K_r
V5F	IISA**F**VGIL	>50	V6D	IISAV**D**GIL	>50	G7A	IISAVV**A**IL	ND
V5L	IISA**L**VGIL	11.1	V6F	IISAV**F**GIL	>50	G7D	IISAVV**D**IL	>50
V5G	IISA**G**VGIL	>50	V6G	IISAV**G**GIL	>50	G7F	IISAVV**F**IL	>50
V5D	IISA**D**VGIL	>50	V6K	IISAV**K**GIL	>50	G7K	IISAVV**K**IL	>50
V5K	IISA**K**VGIL	>50	V6T	IISAV**T**GIL	>50	G7T	IISAVV**T**IL	ND
ML	MLLSVPLLL	1.8				HN654-662	IISAVVGIL	>50

Table I. Most substitutions at P5, P6 and P7 of HN654-662 do not increase binding affinity. K_r is the relative binding constant determined by a T2 cell surface assembly assay. K_r is defined to be the concentration of peptide in μM that yields 50% mean channel fluorescence as compared to the ML peptide. ND not determined. Most substitutions at P5 (V5D, V5G, V5K, and V5T) bind so poorly that even at 50 μM their fluorescence is below the no peptide controls.

As a result, we developed a new hypothesis. This stated that the position 3 residue, which is a secondary anchor, is too small in A2 (it is a serine). We decided to make as series of substitutions at P3 to increase affinity. Once again, we were totally wrong (Table II).

Name	Sequence	K_r	MF
S3	KISAVVGIL	>50	31.2
S3D	KIDAVVGIL	>50	8.4
S3F	KIFAVVGIL	>50	27.8
S3G	KIGAVVGIL	>50	20.2
S3L	KILAVVGIL	>50	9.6
S3T	KITAVVGIL	>50	9.7

Table II. **P3 substitutions do not improve binding affinity**. Description of each is as described in the legend to Table I. MF is maximal fluorescence. This shows that although all the peptides were of relatively low affinity, we could observe differences. Surprisingly, the best appeared to be the wild-type serine at position three.

Single positions don't make high affinity peptides regardless of the position or the identity. Therefore, we come back to our first hypothesis that there are combinations of residues that are bad for HN654-662 binding. To reduce the number of peptides to synthesize, we took

advantage of the fact that the KIFAVVGIL (S3F) peptide in the P3 substitution experiment (Table II) is very similar to KIFGSLAFL the E75 peptide from HER-2/neu that binds with much higher affinity. Therefore, we have ordered the following series of peptides in Table III. This shows a progress from single (2-5), double (6-11) and triple (12-15) amino acids substitutions until the peptide is converted from HN654-662 (S3F) (1) to E75 (16).

GP2	IISAVVGIL	11.7		KIF**G**VV**A**IL	
			KIF10	KIF**G**V**L**GIL	4.4
	KIFAVVGIL			KIFA**S**V**A**IL	
	KIF**G**VVGIL			KIF**GSL**GIL	
	KIFA**S**VGIL			KIFA**SLA**IL	
	KIFAV**L**GIL			KIF**GS**V**A**IL	
	KIFAVV**A**IL			KIF**G**V**LA**IL	
	KIFA**SL**GIL				
KIF8	KIFAV**LA**IL	0.0			

Table III Chimeric peptides from GP2 to E75 made to probe for inappropriate interactions between peptide and MHC or within the peptide itself. MPF is the maximal percentage fluorescence compared to the ML index peptide.

Examination of the peptides that showed improved binding led us to test peptides KIF16 and KIF17. The fact that KIF16 binds signficantly better than any GP2 peptide to date, led us to test KIF18 to test if the phenylalanine at position 8 may be important and KIF 19 and KIF20 to test the roles of the P4 and P6 residues. As can be seen, KIF18 binds significantly better than any other single amino acid substituted peptide. This is curious because the amino acid at this position does not make contacts with the MHC. It is unclear how this amino acid effects binding affinity. We are continuing to investigate this now.

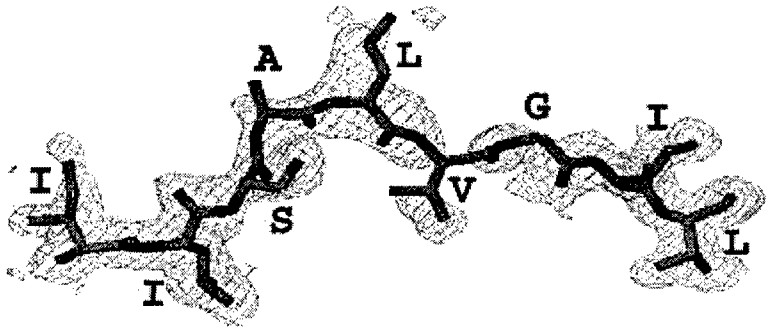
Peptide	Sequence	MPF
KIF16	**KIFASVAFL**	61.9
KIF17	**KIFGSLGFL**	2.2
KIS18	**KI**SAVVG**FL**	54.1
KIF19	**KIFA**SVG**FL**	33.1
KIF20	**KIFA**VV**AFL**	20.2
E75	**KIFGSLAFL**	78.6

Table IV. Probing specific residues identified by amino acids in Table III.

We have also made the following observation. Individual substitutions such as I2L, L9V both increase binding affinity, but not additively. In addition, some combinations of these substitutions clearly interact negatively (Table IV). In our studies of the anchor substituted peptides, we found that the combination of an anchor substitution (I2L/V5L) and a central amino acid substitution (V5L) in combination can result in decreased affinity suggesting that the residues interact. This result is unanticipated as it has been reported that each position is independent (31). We are looking into the structural basis of this now. We believe that the increased binding affinity of V5L is because the leucine binds in a nonpolar pocket under the $\alpha 2$ α helix (Fig 2). However, the structure of the triple substitution, I2L/V5L/L9V (ILSALVGIV), shows the position 5 leucine directed towards solvent. In addition, many researchers alter anchor residues and expect that they don't cause changes to the TcR contacting residues. We determined the crystal structures of I2L/V5L/L9V and I2L/V5L and show that the change of L9V alters the

orientation of the L at P5. This is confirmed by comparing these structures with GP2 bound to HLA-A2. We are confirming the effect on immunogenicity by examining reactivity of the various combinations on I2L-specific T cells now.

Figure 2. **The structure of a triple-substituted, HN654-662-variant peptide, I2L/V5L/L9V, has broken electron density, as does HN654-662.** Averaged omit density (DM) is shown with a 1.5 Å cover radius at a contour of 1 sigma. The A2 molecule has been removed for clarity. The peptide, ILSALVGIV, was folded with A2 *in vitro*, purified and crystallized as described previously (9, 25). The crystallographic data were collected in 2° oscillations on an R-Axis IIC imaging plate system. The data were processed with DENZO and SCALEPACK and the molecular replacement solution determined by AMoRe. Refinement was conducted with CNS. The structure is completed with good statistics.



Name	Sequence	K_r (μM)	T_m (°C)
HN654-662	IISAVVGIL	>50	36.4
I2L	ILSAVVGIL	22.9	42.2
L9V	IISAVVGIV	>50	38.8
I2L/L9V	ILSAVVGIV	10.0	42.5
V5L	IISALVGIL	>50	ND
I2L/V5L	ILSALVGIL	49.3	39.0
V5L/L9V	IISALVGIV	>50	38.8
I2L/V5L/L9V	ILSALVGIV	17.5	39.5

Table V. Affinity measurements for combinations of substitutions show that the stabilizations are not additive. T_m values are the temperature at which 50% of the protein is denatured. K_r is defined in Table I.

D. *Chemically synthesize cysteine mutant sequence and test for binding to HLA-A2 in an in vitro assay. Link to Biacore chip and measure on and off-rates to HLA-A2.1. Complete by 9/97.*

Methods: We will synthesize the peptides through standard FMOC chemistry and will test them for binding as described above.

Progress: We have not begun this aim. We have instead focused on determining why HN654-662 binds poorly and dissecting the interactions between the binding residues.

Specific Aim 2. Screen improved epitopes for enhanced affinity for the T cell receptor.

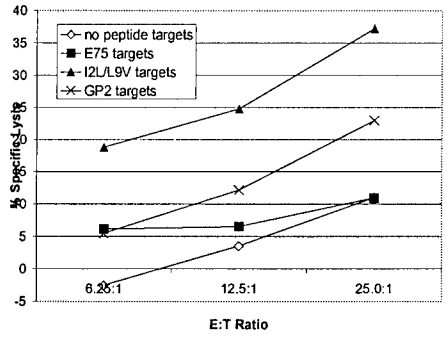
A. Isolate murine CTL lines specific for HN654-662. Line by 12/96 clone by 6/97. Approx. 75 mice.

Methods: CTL are most readily made by stimulation by professional antigen-presenting cells. We will isolate dendritic cells from PBLs of $A2K^b$ mice and stimulate T cells with HN654-662 and altered-peptide ligands. Lines will be generated by repeated in vitro stimulations, clones by limiting dilution or FACS.

Progress: We used human PBLs to generate HN654-662 specific T cells. HLA-A2 donors were obtained from a different project. Dendritic cells from these donors were used to stimulate T cells using HN654-662 (HN654-662). A very weak response to HN654-662 was generated after three stimulations in vitro with human dendritic cells, but it was not readily reproducible and the level of specificity was not high. The T cells did not survive long enough to allow us to clone these cells either.

GP2 did not generate an immune response in A2Kb transgenic mice. Some altered peptides generate an immune response including I2L, but only when given on peptide-pulsed dendritic cells. These data were published in the Journal of Immunology (reprint attached.)

Figure 3. **I2L variants are immunogenic in $A2K^b$ mice**. Mice were immunized as above with I2L/L9V and CTL measured as described above. As can be seen the I2L/L9V immunized mice recognize I2L/L9V pulsed targets significantly better than E75 pulsed peptide (irrelevant peptide), HN654-662 or no peptide targets.



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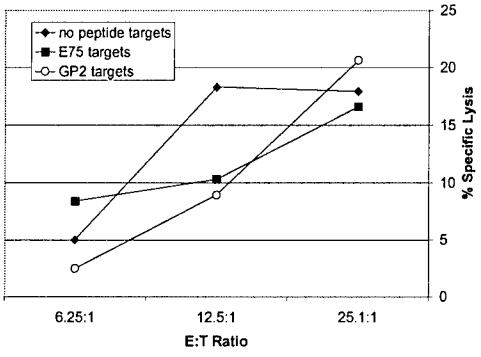

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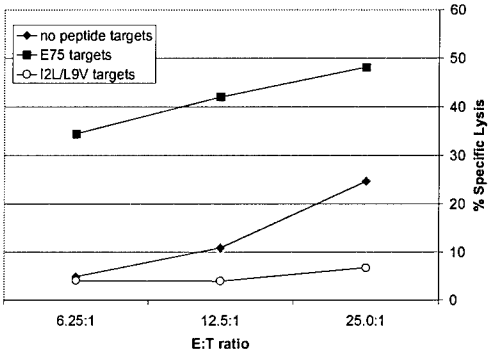

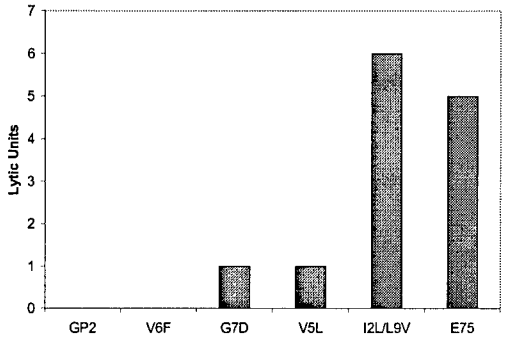

Figure 4. HN654-662 variants at positions 5, 6 and 7 are not immunogenic compared to anchor variants or other HER-2/neu peptides. Immunizations and CTL lysis are as described above. Lytic units are the regression analysis of %specific lysis versus E:T ratio. The number is the number of effectors per 10^6 cells that generate 20% specific lysis.

Dr. Roland Tisch (UNC Microbiology and Immunology) has made mice transgenic for a chimeric $A2K^b$ molecule. We have immunized these mice with HN654-662 and a set of variants to test for immunogenicity. HN654-662 failed to generate a response in these mice regardless of the immunization protocol. In the past year we have immunized with a variety of variant peptides. The question we wished to ask was whether the flexibility in the center of the peptide observed in the crystal structure was detrimental to immunogenicity. To investigate this idea, we are immunizing with some of the variant peptides in Table I that contain the wild-type anchors. We have found that some of these peptides generate an immune response, but it is very weak (Figure 5). The poor immunogenicity is not directly related to the poor affinity because another HER-2/neu peptide F56, is immunogenic. We have also examined the immunogenicity of HER-2/neu peptides in the A2Kbxneu transgenic animals using a vaccinia virus encoding HER-2/neu. As can be seen from Table 5, four out of seven peptides generate a CTL response from peptide immunization and only one (E75) makes a good response when in the context of the entire protein in vaccinia virus. This suggests that mechanisms of immunodominance may play a role in tumor progression.

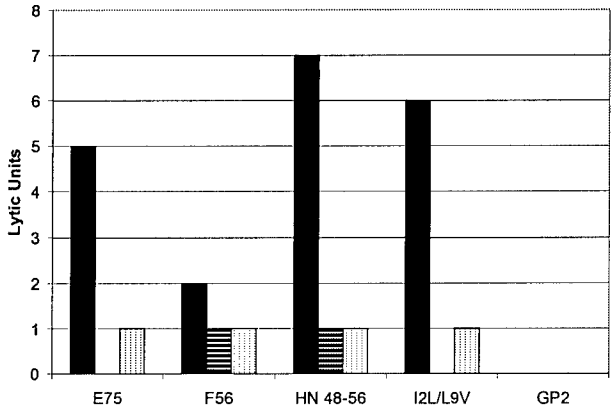

Figure 5. HN654-662 is not immunogenic in $A2K^b$ transgenic mice, but other HER-2/neu-derived peptides are. A. Titration of effector cells shows that the effectors generated by HN654-662 immunization do not recognize HN654-662 better than E75. B. E75 immunized mice show a good specific response. However, another HER-2/neu-derived peptide with similarly poor affinity, F56 (YISAWPDSL), is immunogenic. A variant of HN654-662 with slightly higher affinity, I2L/L9V is also immunogenic. Higher affinity HER-2/neu derived peptides E75 and HN48-56 are also immunogenic. $A2K^b$ mice were immunized 3 times in 10-day intervals after which spleens and popliteal and inguinal lymph nodes were removed. The cells were stimulated with irradiated splenocytes peptide pulsed with the peptides given on the x-axis. C. Lysis data converted to lytic units to give one value describing the number of effectors per 10^6 cells.

Peptide (neu #)	Sequence	$T_{1/2}$	K_r	CTL Peptide	CTL Vaccinia virus
E90 (798)	CLTSTVQLV	7.5	11.4	ND	+/-
E71 (799)	QLMPYGCLL	8.0	2.6	-	-
E75 (369)	KIFGSLAFL	8.6	14.4	+	+
773	VMAGVGSPYV	5.2	15.5	+	+/-
C85 (971)	ELVSEFSRM	ND	>50	-	-
689	RLLQETELV	13.6	14.9	+	-
952	YMIMVKCWMI	ND	ND	ND	ND
48	HLYQGCQVV	13.6	10.1	+	-
F56	YISAWPDSL	ND	>50	-	-

Table VI. HER-2/neu epitopes shown to recognized by CTL from human adenocarcinoma patients common between rat and human HER-2/neu genes. Affinity measurements that have been made have been quoted. $T_{1/2}$ are measured as described previously (Pogue et al). ND not determined. CTL peptide is the whether peptide immunization generates peptide specific T cells. CTL vaccinia virus is whether vaccinia virus encoding rat HER-2/neu generates peptide-specific CTL. + is generates specific CTL reproducibly, +/- is generates CTL at least once in 5 attempts.

B. Transfect LINE1 with HER2/neu and HLA-A2.1 and select. Complete by 12/96
Methods: Using cDNAs for HER-2/neu and HLA-A2/K^b, make stable transformants of LINE1 for inoculation into the Tg A2/K^b mice.

Progress: We have altered the project to examine why the HN654-662 peptide is not immunogenic in mice and looking at other immunogenic peptides from HER-2/neu. In addition, we do not need to transfect LINE1 because A2Kb x neu mice spontaneously generate tumors. We are culturing those tumors now (See Section F).

C. Test CTL reactivity of variant peptide determined from 1 using CTL clones. Complete by 12/97
Methods: Using RMA-S cells or syngeneic B cells, peptide pulse and perform the standard 4 hour chromium release assay on APL.

Progress: As described above, we have switched our efforts to examining why HN654-662 does not bind well and why it is not immunogenic. We now have the A2K^b mouse. We cannot however generate T cells towards HN654-662. If we are able to generate a good response with one of the variants that recognizes HN654-662 also, we will clone those T cells and perform this aim.
Methods: We could not previously use the mice of choice, the mice that are transgenic for HLA-A2/K^b are presently on the wrong genetic background. We had altered our plans to use human T

cells and test reactivity against established tumor cell lines and primary tumors. However, we were not successful at reproducibly generating a response to HN654-662 from human PBMC. Since we now have the mice required, and the question has shifted slightly, we are again working in the $A2K^b$ mice on the FVB background. $A2K^b$ mice were immunized with the peptide substitutions shown in Table I and the immune response has and continues to be tested. Figure 1 shows that responses can be made to I2L/L9V, but not HN654-662 or V5L suggesting that the affinity of HN654-662 is just too low to generate a primary immune response. However, an equally poor binding HER-2/neu derived peptide (F56) is able to generate a good response.

E. Produce soluble TCR. Complete by 6/98.

Methods: Isolate cDNA using Fast-Track kit (Invitrogen). Clone and determine sequence. Using PCR make constructs for expression in the baculovirus system and also as fusion proteins of variable domains using the TrcThioHis system (Invitrogen). Isolate by metal chelation chromatography and other chromatographic methods as required. Test for proper folding using monoclonal antibodies that recognize the corresponding alpha and beta chains.

Progress: We have put this aim on hold until we identify an APL that generates a good response to HN654-662.

F. Test affinities of class I/ peptide complexes with soluble TCR. Complete by 12/98.

Methods: Surface plasmon resonance will be used to measure on and off rates of complexes fixed to the surface of appropriate chips. In each case, we will engineer E. coli BirA recognition sites to specifically biotinylate the carboxyl terminus of each protein. Then each protein individually will be bound to strepavidin-coated chips. On and off rates should be independent of which protein is coupled to the chip.

Progress: We have successfully engineered BirA recognition sequences to the carboxyl end of HLA-A2.1. These proteins fold in vitro as well as the wild-type A2 sequences. We will perform the same to the T cell clone isolated. We have also successfully cloned a different TcR and testing our abilities to produce the protein when we get a HN654-662-specific T cell clone. We have successfully generated E75-specific T cell clones. We have folded the AHIII12.2 and P14 TcR in vitro and shown that they bind to their respective peptide/MHC combinations by Biacore. We are immunizing with I2L to generate GP2-specific T cells now.

G. Test class I peptides identified in 1 as vaccines with transgenic mice. Complete by end of grant.

Methods: Peptide-pulsed dendritic cells (DCs) will be used as vaccines to generate specific T cell responses. The DCs may be used to test protection in a tumor challenge model or tested for their ability to slow or reduce the growth of a tumor.

Progress: In collaboration with Dr. Roland Tisch, we now have a good tumor challenge model. We have crossed the $A2K^b$ mice onto the rat HER-2/neu tg mouse to get an $A2K^b$ x neu mouse. The rat neu gene is under control of a mouse mammary tumor virus promoter. These mice spontaneously develop mammary tumors at approximately 5 months of age. We have begun to examine the T cell response to HER-2/neu peptides bound to A2Kb. Unfortunately, HN654-662 is not common between human and rat protein sequences so we cannot use this model system to study HN654-662. We are using to study other low affinity peptides from HER-2/neu that are common between the human and rat proteins. We are able to generate a good CTL response

from many of these peptides. We do not know if these responses will be protective at this time. We are culturing the tumors to generate tumor lines that may be used in a tumor challenge situation.

6. Key Research Accomplishments

- Determined crystallographic structure of A2/HN654-662.
- Measured binding to large numbers of peptide variants that do not significantly increase affinity.
- Determined structure of A2/I2L/V5L/L9V and A2/I2L/V5L
- Designed peptides with increased affinity that when used to immunize mice generate CTL that react with HN654-662.
- Measured CTL responses from $A2K^b$ x neu mice to low and intermediate affinity peptides presented by the tumors. This suggests that developing anti-tumor CTL is a viable technique.
- Showed that I2L variant is immunogenic in mice when pulsed on dendritic cells. This peptide is now in a clinical trial in collaboration with Dr. Jon Serody UNC Lineberger Comprehensive Cancer Center.
- Showed that tumor progress may be related to immunodominance mechanisms in that the epitopes recognized depend on whether they are presented as peptides or proteins.
- Determined that the P8 residue of GP2 plays a role in binding affinity when it doesn't interact with MHC.
- Determined that each amino acid does not interact independently when bound to class I MHC.

7. Reportable Outcomes

Manuscripts published:

Kuhns, J. J., Batalia, M. A., Yan, S., and Collins, E. J. (1999) Poor Binding of HER2/neu Epitope to HLA-A2.1 is Due to Lack of Interactions in the Center of the Peptide, *J. Biol. Chem 274: 36422-36427.*

Batalia, M.A, Kirksey, T.J., Sharma, A., Jiang, L., Zhao, R., and Collins, E. J. (2000) "Class I MHC is Maximally Stabilized to Thermal Denaturation at Physiological NaCl Concentration" *Biochemistry* 39, 9030-9038.

Serody, J., Collins, E. J., Tisch, R., Kuhns, J. J. and Frelinger, J. A., (2000) "Generation of T-cell Reactivity to Self-Proteins with Professional Antigen Presentation Cells" *J. Immunol.* 164 4961-4967.

Manuscript submitted:

Sharma, A., Kuhns, J.J. and Collins, E.J. (2000) "Class I MHC Anchor Substitutions Alter the Conformation of T Cell Receptor Residues" (submitted).

We have applied for and received a Susan B. Komen Breast Cancer Foundation Research Grant based on this work.

8. Conclusions:

We have made significant progress within the last year and over the course of the grant. We have had three manuscripts published and are presently writing three others. We have used the information found to design peptides that have improved binding to class I MHC. We have tested many more that do not have increased binding affinity. We are close to understanding the poor binding affinity of HN654-662. We have found a new phenomenon of interaction between the binding residues in the peptide. We have a good animal model to test for immunogenicity of these peptides. We have redesigned the experimental scheme to examine T cell recognition in transgenic mice. We now have all the tools at hand and the experiments are in full swing in order to generate a potent immunotherapeutic.

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10. Appendices:

TNF Receptor 2-Deficient CD8 T Cells Are Resistant to Fas/Fas Ligand-Induced Cell Death[1]

Hung-Sia Teh,[2] Andrew Seebaran, and Soo-Jeet Teh

Apoptotic cell death plays a fundamental role in the maintenance of tissue homeostasis in complex biological systems. It is also a major mechanism for keeping immune reactions in check. Members of the TNF family of receptors and cytokines are implicated in the regulation of apoptotic signals that shape the immune system. In this study, we have examined the role of three members of the TNFR family, Fas (CD95), TNFR1 (p55), and TNFR2 (p75), in inducing cell death in Con A-activated CD4 and CD8 T cells. It was found that Con A-activated $p55^{-/-}$ CD4 or CD8 T cells were highly resistant to TNF-induced cell death. By contrast, although activated $p75^{-/-}$ CD4 or CD8 T cells were killed by TNF, they were more resistant to TNF-induced killing when compared with $p75^{+/+}$ cells, particularly at higher concentrations of TNF. We also determined whether activated $p55^{-/-}$ and $p75^{-/-}$ T cells differ in their sensitivity to cell death induced by TCR cross-linking. We found that activated $p55^{-/-}$ CD4 or CD8 T cells were equally susceptible to TCR-induced cell death. More interestingly, the loss of the p75 receptor conferred resistance to TCR-induced death in activated CD8, but not CD4 T cells. This resistance to TCR-induced death in activated $p75^{-/-}$ CD8 T cells correlated with the resistance of these cells to Fas/Fas ligand-induced cell death. *The Journal of Immunology,* **2000, 165: 4814–4821.**

Fas (CD95) and its ligand (FasL)[3] are members of the TNFR and TNF families, respectively (1, 2). This receptor-ligand pair plays an important role in the homeostasis of the peripheral immune system (3). FasL is expressed predominantly on activated lymphocytes and is able to induce programmed cell death on virtually all Fas-expressing cells, if they are receptive to its signal (3, 4). The importance of this interaction for the maintenance of lymphocyte homeostasis is demonstrated in the generalized lymphoproliferative disorder associated with natural loss-of-function mutations of Fas (*lpr*) and its ligand (*gld*) (2, 5). Mice homozygous for *lpr* or *gld* develop remarkably similar progressive nonmalignant lymphoproliferative diseases characterized by splenomegaly, severe lymphadenopathy, hypergammaglobulinemia, circulating autoantibodies, and premature death.

TNF, the prototype member of this family, is also potentially important in the induction of programmed cell death (1). This cytokine is a product of many cell types, but particularly of leukocytes, and is an important mediator in the early stages of the inflammatory response (1). Experiments with gene knockout mice have also shown that the two TNFRs, TNFR1 (p55) (6) and TNFR2 (p75) (7), participate in the induction of apoptosis and subsequent deletion of Ag-specific mature T cells. However, $p55^{-/-}$ and $p75^{-/-}$ mice are normal with respect to size and the composition of their lymphocyte compartments (8, 9). Both Fas and TNFR1 contain conserved death domains in their cytoplasmic tails, which mediate defined protein-protein interactions (10, 11), allowing the recruitment of other death domain-containing proteins such as Fas-associated death domain protein (FADD) (MORT1), TNFR-associated death domain protein, or receptor interacting protein (RIP) (12–17). The association of FADD to Fas or TNFR1 results in the recruitment of Fas-associated death domain-like IL-1-converting enzyme/MORT1-associated CED-3 homologue (caspase 8), the activation of which in turn leads to cell death (18, 19). In contrast to Fas or TNFR1, the cytoplasmic tail of TNFR2 does not contain a death domain and does not interact with death domain-containing proteins such as FADD, RIP, or TNFR-associated death domain protein. However, several studies have shown that TNFR2 can also transduce TNF-dependent apoptotic signals (7, 20). More recently, it was shown that cell death mediated by TNFR2 is dependent on the presence of RIP, a protein Ser/Thr kinase previously shown to be required for NF-κB activation by TNFR1 (21). These studies suggest that other signaling molecules mediate the induction of cell death by this receptor. The cytoplasmic tail of TNFR2 has been shown to interact with signal transduction molecules known as TNFR-associated factor (TRAF) proteins, particularly TRAF1 and TRAF2 (22). In some cases, the TRAF proteins have been shown to mediate various biological effects exerted by their cognate receptors. For example, TRAF2 was shown to mediate NF-κB activation (23–25).

In this study, we have used Con A-activated CD4 and CD8 T cells from *lpr/lpr*, $TNFR1^{-/-}$, or $TNFR2^{-/-}$ mice to determine the relative contribution of the TNF and Fas signaling pathways in inducing cell death in these cells. Our findings are consistent with the hypothesis that TNFR2 plays distinct regulatory roles in cell death induced by TNF or FasL in activated CD4 and CD8 T cells.

Materials and Methods

Mice

Breeders for C57BL/6 (B6), B6-$p55^{-/-}$, B6-$p75^{-/-}$, and B6-*lpr/lpr* mice were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice

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[3] Abbreviations used in this paper: FasL, Fas ligand; 7-AAD, 7-amino actinomycin D; FLIP, Fas-associated death domain-like IL-1-converting enzyme-inhibitory protein; c-FLIP, cellular FLIP; c-IAP, cellular inhibitor of apoptosis protein; FADD, Fas-associated death domain protein; RIP, receptor interacting protein; TRAF, TNFR-associated factor.

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were bred at the Animal Unit in our department. Mice of 6–8 wk of age were used for the experiments described.

Abs and flow cytometry

Abs and their specificities were as follows: Jo2, Fas (CD95) (PharMingen, San Diego, CA); K10, Fas ligand (PharMingen); 53.67, CD8α (American Type Culture Collection (ATCC), Manassas, VA); 53.58, CD8β (ATCC); GK1.5, CD4 (ATCC); 145-2C11, CD3ϵ (ATCC); 55R-593, p55 (Genzyme, Cambridge, MA); TR75-32, p75 (Genzyme). A FACScan equipped with the LYSYS II software (Becton Dickinson) was used to acquire and analyze the data. For three-color analysis, a total of 25,000 events was acquired.

Cell cultures

$CD4^+CD8^-$ (CD4) and $CD4^-CD8^+$ (CD8) T cells were isolated from the lymph nodes of the indicated mouse lines by incubating the cells with biotinylated anti-CD4 or anti-CD8β mAb, respectively, followed by positive selection using a MACS MS^+ Separation Column and MiniMACS magnet following the procedure provided by the manufacturer (Miltenyi Biotech, Auburn, CA). The purified CD4 or CD8 lymph node cells were of >95% purity, and 5×10^5/ml of purified cells were cultured with 2.5 μg/ml of Con A and 20 U/ml of IL-2 for 48 h. All cells were cultured in Iscove's DMEM (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% (v/v) FBS and antibiotics (I-medium). The cultures were then split 1 in 2 in I-medium containing 20 U/ml of IL-2 and cultured for an additional 24 h. After this culture period, the cells were assessed for the expression of cell surface markers or in TCR-induced cell death assays, as indicated. For assessment of TCR-induced cell death, day 3 activated cells (1×10^5) were plated onto flat-bottom microtiter wells that had been precoated with 1 μg/ml of 2C11 mAb in 0.20 ml of I-medium containing 20 U/ml of IL-2. After an incubation period of 16 h, the cells were collected from individual wells and assessed for cell death. This was done by incubating the cells with 7-amino actinomycin D (7-AAD; Calbiochem, La Jolla, CA), as previously described (26, 27). The cells were then fixed with paraformaldehyde and analyzed by FACS. Cells staining 7-AAD^- were considered nonapoptotic and viable. The percentage of specific kill was determined as $100 \times (1 - (\%$ of viable cells cultured with 2C11)/(% of viable cells that were cultured without 2C11)).

Lysis of Fas-expressing T cells by $FasL^+$ fibroblasts

The 3T3 fibroblasts that have been mock transfected (3T3-PSRγ) or Fas ligand transfected (FasL-3T3) were kindly provided by Dr. Nick Crispe (Yale University, New Haven, CT). Expression of FasL by the 3T3-PSRγ and the FasL-3T3 cell lines was determined by FACS analysis. For assessment of Fas/FasL-mediated killing, day 3 CD4 or CD8 Con A blasts from various mouse lines were ^{51}Cr labeled. A total of 1×10^4 ^{51}Cr-labeled cells was plated onto wells containing either 3×10^4, 1×10^4, or 3×10^3 3T3-PSRγ or FasL-3T3 cells and incubated for 6 h at 37°C. Spontaneous release was determined by incubating ^{51}Cr-labeled Con A blasts for the same time period in the absence of fibroblasts. Percentage of specific kill was determined as $100 \times$ ((counts released with fibroblasts − spontaneous release)/(maximum release − spontaneous release)). Maximum release was determined by freezing and thawing the ^{51}Cr-labeled Con A blasts three times and determining the radioactivity released into the culture supernatant.

Results

Activated $p75^{-/-}$ CD8 T cells are resistant to TCR-induced cell death

Cross-linking of the TCR on activated T cells leads to programmed cell death. This form of programmed cell death is referred to as propiocidal apoptosis and is dependent on culturing the activated T cells in IL-2 (28, 29). Propiocidal apoptosis is dependent at least in part on the expression of Fas on the activated T cells (30). To determine whether p55 and p75 play any role in propiocidal apoptosis, we activated purified CD4 and CD8 lymph node T cells from B6, B6-*lpr/lpr*, B6-$p55^{-/-}$, and B6-$p75^{-/-}$ mice with Con A and IL-2. Propiocidal apoptosis was induced by TCR cross-linking. The 7-AAD assay (see *Materials and Methods*) was used for distinguishing live from dead cells. The results in Fig. 1*A* indicate that Con A-activated CD4 T cells from B6 and B6-$p75^{-/-}$ mice were highly susceptible to propiocidal apoptosis. As expected, activated CD4 T cells from B6-*lpr/lpr* mice were relatively resistant

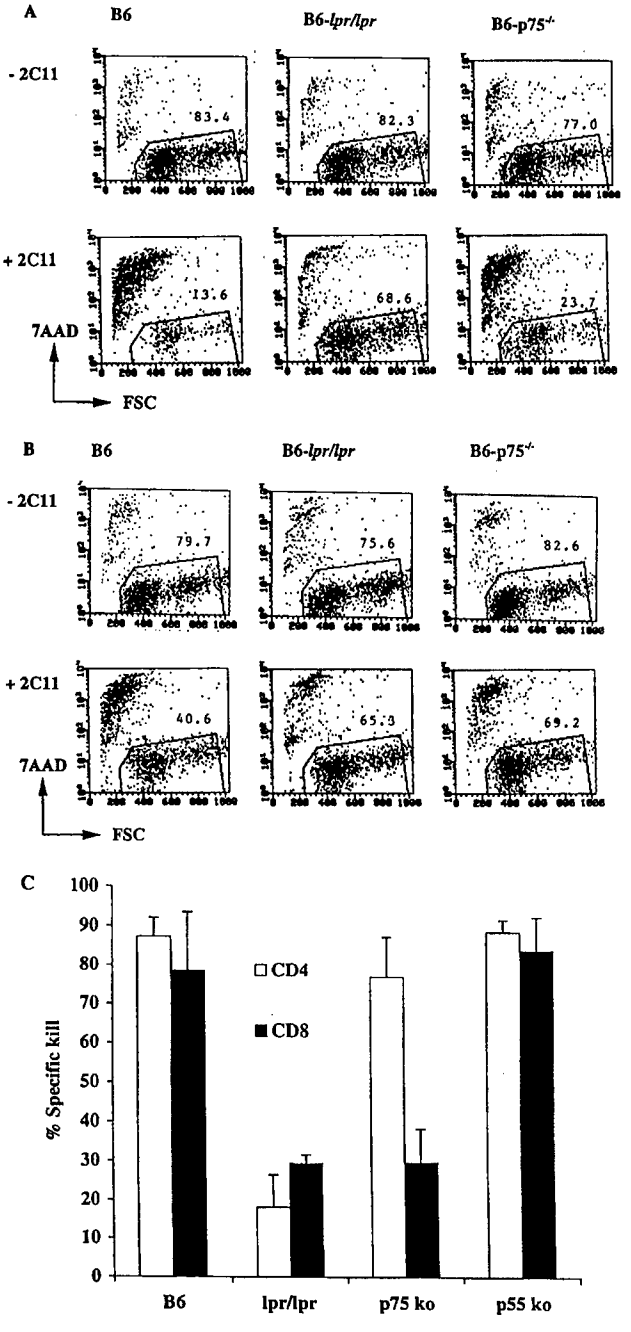

FIGURE 1. CD8 Con A blasts from $p75^{-/-}$ mice are resistant to TCR-induced cell death. Day 3 Con A blasts from the indicated mouse line were incubated with and without immobilized 2C11 mAb and IL-2 for 16 h at 37°C. IL-2 was included in these cultures to prevent cell death as a result of IL-2 deprivation. After this incubation period, the cells from individual cultures were collected and stained with 7-AAD and analyzed by FACS. Dot plots of 7-AAD fluorescence vs forward scatter (FSC) for CD4 (*A*) and CD8 (*B*) Con A blasts after the 16-h incubation period are indicated. The numbers in *A* and *B* indicate the percentage of viable cells after the 16-h incubation period. Percentage of specific kill (*C*) is calculated as described in *Materials and Methods*. The error bars are SDs of triplicate cultures.

to propiocidal apoptosis (Fig. 1*A*). Activated CD8 T cells from B6 and B6-*lpr/lpr* mice were similar to CD4 T cells in their susceptibility and resistance to propiocidal apoptosis, respectively (Fig. 1*B*). Unexpectedly, activated CD8 T cells from B6-$p75^{-/-}$ mice were highly resistant to propiocidal apoptosis (Fig. 1*B*). These results are also expressed as percentage of specific kill (Fig. 1*C*) because this form of data presentation offers a more quantitative

way for comparing results between the various groups. We also found that similar to cells from B6 mice, activated CD4 and CD8 T cells from B6-p55$^{-/-}$ mice were also highly susceptible to propiocidal apoptosis (Fig. 1*C*). Therefore, only the p75$^{-/-}$, and not the p55$^{-/-}$ mutation confers resistance of activated CD8 T cells to propiocidal apoptosis.

Because TCR-induced cell death is mediated in large part by Fas/FasL interactions, we determined whether the resistance of activated p75$^{-/-}$ CD8 T cells to TCR-induced cell death could be due to alteration in Fas expression by these cells. The results in Fig. 2 indicate that Fas expression in Con A-activated CD4 and CD8 T cells is not affected by the p75$^{-/-}$ mutation. Similarly, the expression of p75 in Con A-activated CD4 and CD8 T cells is not affected by the *lpr* mutation. The fluorescence data also indicate that CD4 and CD8 Con A blasts from B6-*lpr/lpr* and B6-p75$^{-/-}$ mice did not express detectable level of Fas and p75, respectively.

Activated p75$^{-/-}$ CD8 T cells are resistant to FasL-induced cell death

Propiocidal apoptosis is also dependent on the induction of FasL in activated T cells as a result of TCR cross-linking (31–35). Therefore, one potential explanation for the resistance of activated p75$^{-/-}$ CD8 T cells to propiocidal apoptosis is that these cells do not up-regulate FasL upon TCR cross-linking. To determine whether activated p75$^{-/-}$ CD8 T cells can up-regulate FasL as a result of TCR cross-linking, Con A-activated CD8 T cells from B6, B6-*lpr/lpr*, and B6-p75$^{-/-}$ mice were stimulated with an anti-CD3ϵ mAb for 6 h, and the induction of FasL expression on these cells was quantitated by flow cytometry. The 6-h incubation time is insufficient for most cells to undergo propiocidal apoptosis and offers a suitable time point for assessment of FasL expression. The results in Fig. 3 indicate that p75$^{-/-}$ CD8 T cells were able to up-regulate FasL at least as efficiently as B6 CD8 T cells after TCR stimulation. Thus, the resistance of p75$^{-/-}$ CD8 T cells to propiocidal apoptosis is not due to the inability of these cells to up-regulate FasL after TCR stimulation. We also noted that B6-*lpr/lpr* CD8 T cells were the most efficient in expressing FasL after TCR stimulation, an observation previously reported by other investigators (36).

The observation that FasL induction is not defective in p75$^{-/-}$ CD8 T cells raises the possibility that these cells are resistant to cell death mediated by Fas/FasL interactions. To test this possibility, we determined the extent of lysis that was caused by incubating activated T cells with a FasL-expressing fibroblast cell line in a ^{51}Cr release assay. The level of FasL expression in the parental fibroblast cell line and the FasL transfectant is shown in Fig. 4*A*. It is clear from this figure that FasL expression is only detectable in the transfectant cell line. The data in Fig. 4*B* showed that CD4 Con A blasts from B6 and B6-p75$^{-/-}$ mice were equally susceptible to killing by the FasL$^+$ cell line. As expected, CD4 blasts from B6-*lpr/lpr* mice were resistant to killing by the FasL$^+$ cell line. By contrast, CD8 blasts from B6-p75$^{-/-}$ mice were much more resistant than CD8 blasts from B6 mice to killing by the FasL$^+$ cell line (Fig. 4*C*). This result suggests that the resistance of CD8 blasts from B6-p75$^{-/-}$ mice to propiocidal apoptosis is most likely due to the resistance of this cell population to Fas/FasL-mediated killing.

Activated p75$^{-/-}$ CD4 and CD8 T cells are more resistant to killing by TNF

Previous studies have suggested that TCR-induced apoptosis in activated T cells is mediated in part by TNF and killing by TNF is mediated by the p75 receptor (7). The availability of B6-p55$^{-/-}$ and B6-p75$^{-/-}$ mice allows us to determine more directly whether TNF-induced killing of CD4 and CD8 Con A blasts is mediated by either the p55 and/or p75 receptor. The sensitivity of CD4 and CD8 Con A blasts to killing by TNF was determined by culturing these blasts with various concentrations of TNF in the presence or absence of a low concentration (1 μg/ml) of the protein synthesis inhibitor, cycloheximide. Previous studies have shown that the addition of cycloheximide is necessary for the apoptotic effects of TNF (37, 38). We also found that between 0.1 and 10 ng/ml, TNF by itself does not induce apoptosis in either CD4 or CD8 blasts from these three lines of mice (data not shown). In the presence of cycloheximide, CD4 or CD8 Con A blasts from B6 mice were highly sensitive to killing by TNF (Fig. 5). At the concentrations tested, CD4 or CD8 blasts from p55$^{-/-}$ mice were completely resistant to killing by TNF plus cycloheximide (Fig. 5). Although CD4 or CD8 blasts from p75$^{-/-}$ mice were susceptible to killing by TNF plus cycloheximide, the dose-response curve to TNF differs from that observed for the corresponding blasts from B6 mice. At the lowest concentration of TNF (0.1 ng/ml), there was no

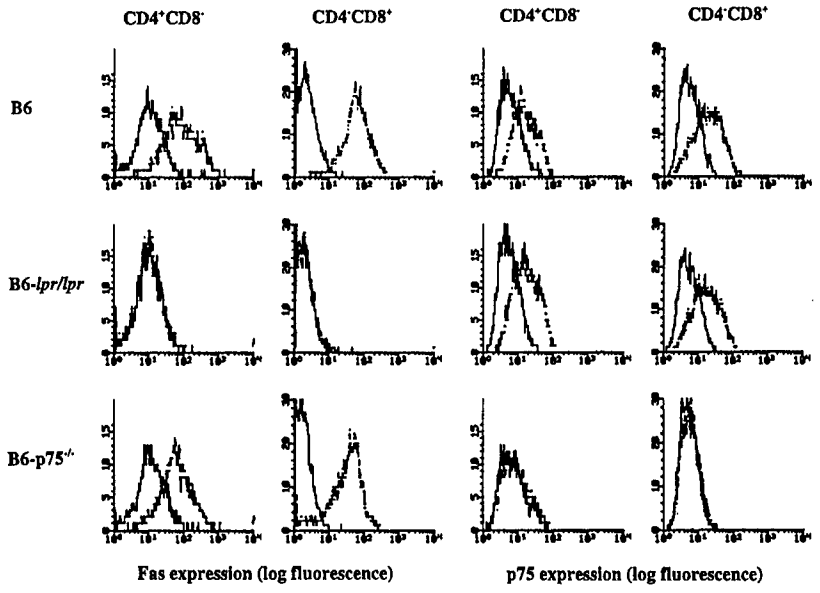

FIGURE 2. Fas (CD95) and TNFR2 (p75) expression on Con A blasts. Purified CD4 or CD8 T cells from the indicated mouse line were activated with Con A and IL-2 for 3 days. The level of Fas expression on these cells was determined by staining with biotinylated anti-Fas (Jo2) mAb, followed by streptavidin-Tricolor. The level of p75 expression was determined by staining the cells with unlabeled anti-p75 mAb, followed by anti-mouse Ig FITC. The solid lines indicate the base level of Fas or p75 expression obtained by staining of the indicated cells with either streptavidin-Tricolor (for Fas expression) or by anti-mouse Ig FITC (for p75 expression).

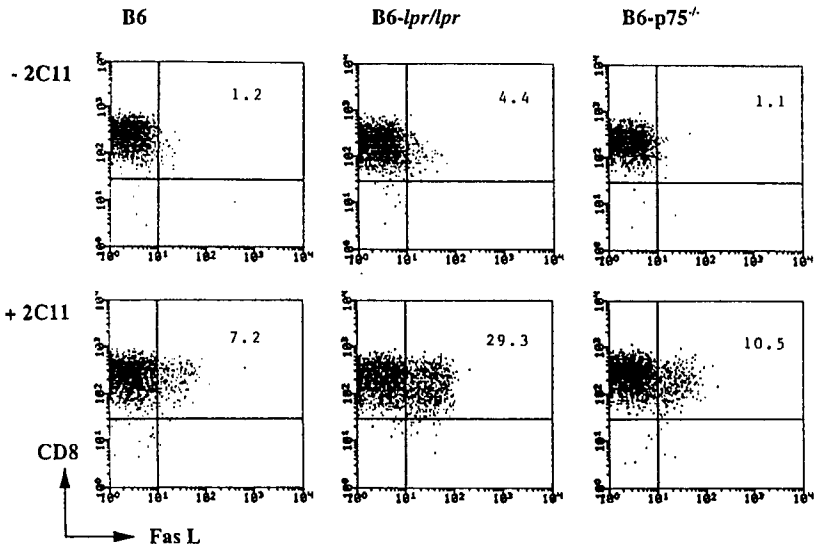

FIGURE 3. Normal induction of FasL in TCR-stimulated $p75^{-/-}$ CD8 T cells. Day 3 CD8 Con A blasts from the indicated mouse line were stimulated with immobilized 2C11 mAb and IL-2 for 6 h at 37°C. The cells were then recovered and stained with biotinylated anti-FasL mAb, followed by streptavidin-Tricolor. The numbers indicate percentages of cells in the quadrant.

difference in susceptibility to killing between CD4 and CD8 blasts from B6 or B6-$p75^{-/-}$ mice. However, at higher concentrations of TNF (1 and 10 ng/ml), CD4 and CD8 blasts from $p75^{-/-}$ mice were more resistant than those from B6 mice to killing by TNF plus cycloheximide. These results indicate that expression of the p75 receptor is insufficient for TNF-mediated apoptosis in both CD4 and CD8 blasts. Furthermore, the absence of the p75 receptor in these blasts renders them less susceptible to killing at high concentrations of TNF.

The p75 receptor is required for the optimal activation of CD4 and CD8 T cells

The results in Fig. 5 suggest that the p75 receptor is incapable of transducing TNF-mediated death signals in CD4 and CD8 Con A blasts. Other studies have suggested that the p75 receptor is required for optimal proliferation of thymocytes and cytotoxic CD8 T cells (39). To further define the function of the p75 receptor in CD4 and CD8 T cells, we determined whether the p75 receptor is required for the optimal proliferative response of CD4 and CD8 T cells in response to TCR stimulation. This was done by purifying CD4 and CD8 T cells from the lymph nodes of B6 and B6-$p75^{-/-}$ mice and activating them with an anti-TCR mAb plus IL-2 and measuring their proliferative response 2 days later. The data in Fig. 6 indicate that the proliferative responses of purified CD4 and CD8 T cells from B6-$p75^{-/-}$ mice in response to anti-TCR stimulation were about 2-fold less than that observed for corresponding cells from B6 mice. By contrast, the proliferative responses of CD4 and CD8 T cells from B6-$p55^{-/-}$ mice in response to stimulation by anti-TCR mAb + IL-2 were similar to that of CD4 or CD8 T cells from B6 mice (data not shown). Thus, the p75, but not the p55, receptor is required for optimal proliferative responses of anti-TCR-stimulated CD4 and CD8 T cells.

Cell recovery from cell cultures at various times after TCR stimulation is affected by both the rate of cell proliferation and cell death. Because we have observed that activated CD8 T cells from B6-$p75^{-/-}$ mice were more resistant to TCR-induced cell death, the expectation is that even though anti-TCR-stimulated $p75^{-/-}$ CD8 T cells proliferated less optimally than B6 CD8 T cells, the higher resistance of activated $p75^{-/-}$ CD8 T cells to TCR-induced cell death will confer a survival benefit, which may be reflected by higher cell recoveries at later time points of the culture. To test this possibility, we stimulated unpurified spleen cells from B6 and B6-$p75^{-/-}$ mice, which contained a mixture of CD4 and CD8 T cells, with anti-TCR mAb plus IL-2 and determined the number of CD4 and CD8 T cells recovered at various times after culture initiation. The data in Fig. 7 show that fairly similar number of CD8 T cells were recovered from anti-TCR-stimulated during the first 3 days of culture. However, on day 4, significantly more number of CD8 T cells were recovered from cultures of $p75^{-/-}$ cells relative to B6 cultures. By contrast, fewer CD4 T cells were recovered from $p75^{-/-}$ cultures relative to B6 cultures, and this lower recovery was evident by day 3 after TCR stimulation. The increase in yield of $p75^{-/-}$ CD8 cells after 4 days of culture was also reflected in the CD8/CD4 ratios of the cultured cells. Before culture, the CD8/CD4 ratios of spleen cells from B6 and $p75^{-/-}$ mice were 0.58 and 0.50, respectively. After 4 days of culture, the CD8/CD4 ratio was 2.3 for B6 and 4.1 for $p75^{-/-}$ spleen cells. These observations are consistent with our conclusions that the absence of the p75 receptor confers a survival advantage for CD8, but not CD4, T cells with regard to TCR-induced cell death.

Discussion

The role of TNFR1 (p55) and TNFR2 (p75) in TNF-induced killing of activated CD4 and CD8 T cells

In the presence of low concentrations of cycloheximide, activated CD4 and CD8 T cells are killed by TNF (Fig. 5). Under our assay conditions, TNF-mediated killing of these cells is dependent on two factors: 1) the presence of the p55 receptor, and 2) the inclusion of cycloheximide in the killing assay. Zheng et al. (7) have previously reported that the killing of activated CD8 T cells by TNF is mediated by the p75 receptor. The authors' conclusion was based in part on the failure to detect expression of the p55 receptor on activated CD4 and CD8 T cells. We have also been unable to detect expression of the p55 receptor on activated CD4 and CD8 T cells by FACS analysis (data not shown). However, the abrogation of killing by TNF plus cycloheximide in activated $p55^{-/-}$ CD4 or CD8 T cells (Fig. 5) indicates that p55 is essential for this process. Therefore, p55 must be expressed on these cells at a level that is below detection by FACS analysis. Our data also indicate that TNF is unable to induce killing of either activated CD4 or CD8 T cells via the p75 receptor because activated $p55^{-/-}$ CD4 or CD8 T cells express the same level of p75 as activated cells from B6 mice (data not shown) and these cells are resistant to killing by TNF plus cycloheximide (Fig. 5). Speiser et al. (6) have also shown that the p55 receptor is required for the deletion of activated CD8 cytotoxic

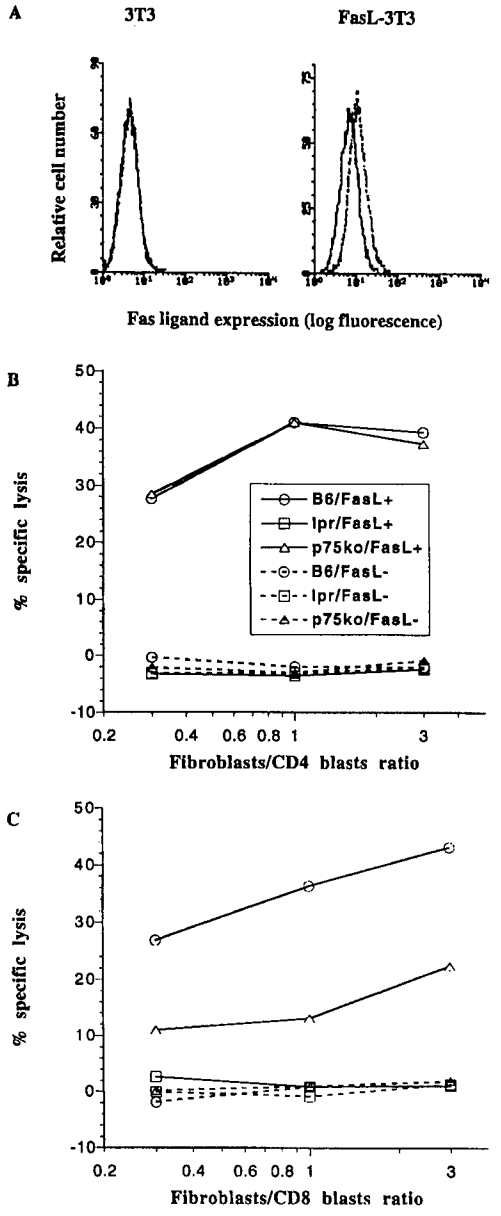

FIGURE 4. CD8, but not CD4, Con A blasts from $p75^{-/-}$ mice are resistant to Fas/FasL-mediated killing. Day 3 CD4 or CD8 Con A blasts from the indicated mouse line were ^{51}Cr labeled and incubated either with $FasL^-$ (3T3-PSRγ) or $FasL^+$ (FasL-3T3) fibroblasts. *A*, The level of FasL expression on these cell lines was determined by staining the cells with a biotinylated anti-FasL mAb, followed by streptavidin-Tricolor. Only FasL-3T3 fibroblasts express detectable level of FasL. *B*, The ratios of fibroblasts to CD4 Con A blasts are as indicated. IL-2 was included in the assay medium to prevent cell death resulting from IL-2 deprivation. After a 6-h incubation period, the amount of ^{51}Cr release into the culture supernatant was determined. The percentage of specific lysis values are the mean of duplicate cultures. *C*, Similar to *B*, except CD8 Con A blasts from the indicated mouse lines were substituted for CD4 Con A blasts.

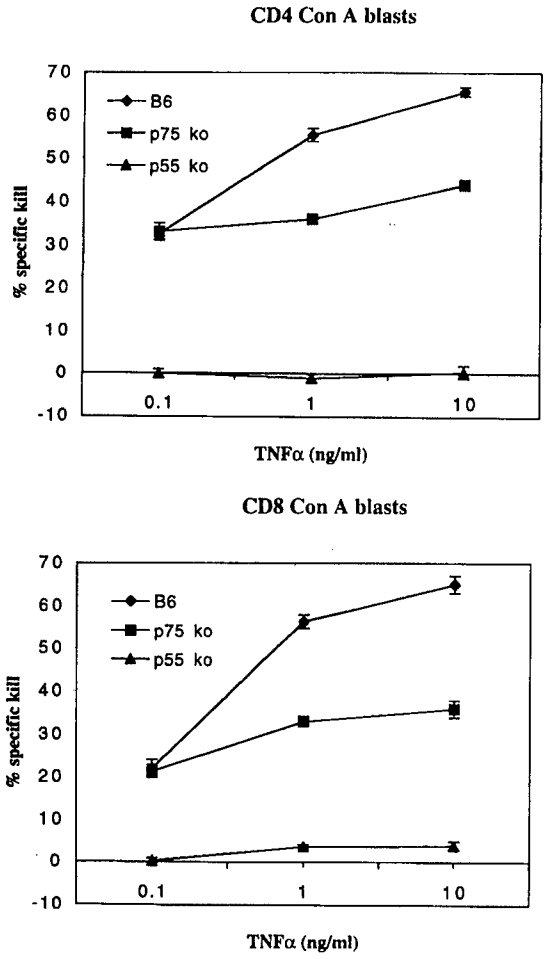

FIGURE 5. TNF-induced cell death in activated CD4 or CD8 cells is dependent on p55 expression and is regulated by p75. Day 3 CD4 or CD8 Con A blasts from the indicated mouse line were cultured with the indicated concentration of TNF, 20 U/ml of IL-2, and 1 μg/ml of cycloheximide for 16 h at 37°C. Percentage of specific kill is calculated as described in *Materials and Methods*. The error bars denote SDs of triplicate cultures. In the absence of cycloheximide, little or no TNF-induced killing can be detected (data not shown).

T cells in vivo. We found that the role of p75 is to augment TNF-induced, p55-mediated killing at high concentrations of TNF (Fig. 5). The augmentation of TNF-induced, TNFR1-mediated cell death by TNFR2 has also been observed by Weiss et al. (37). Previous studies have shown that TNFR1 can mediate both apoptotic and antiapoptotic signals (15, 40). Weiss et al. (37) proposed that the augmentation of TNF/TNFR1-mediated cell death by TNFR2 may be due to the sequestration of antiapoptotic proteins by the p75 receptor complex. p75 may also augment TNF/TNFR1-mediated killing of CD4 and CD8 T cells by a similar mechanism.

The role of p75 in TCR- and Fas-mediated killing of activated CD4 and CD8 T cells

We found that TCR-mediated killing of activated CD8, but not CD4, T cells is drastically reduced in the absence of the p75 receptor (Fig. 1). Zheng et al. (7) proposed that there are two effector mechanisms associated with cell death induced by cross-linking the TCR on activated T cells. The first is the induction of FasL in activated T cells as a result of TCR cross-linking, and this can lead to activation of the Fas death pathway as a result of Fas-FasL interaction. The second mechanism is that TCR stimulation of activated T cells leads to the production of TNF, which initiates the TNF-dependent death pathway. Zheng et al. (7) proposed that the Fas/FasL-mediated pathway is sufficient for TCR-induced killing of activated CD4 T cells. They also proposed that Fas/FasL-mediated killing of activated CD8 T cells is a minor pathway, and the major pathway for TCR-induced cell death of activated CD8 T cells is mediated by a p75-dependent TNF pathway. Our data are inconsistent with these conclusions for the following reasons: 1)

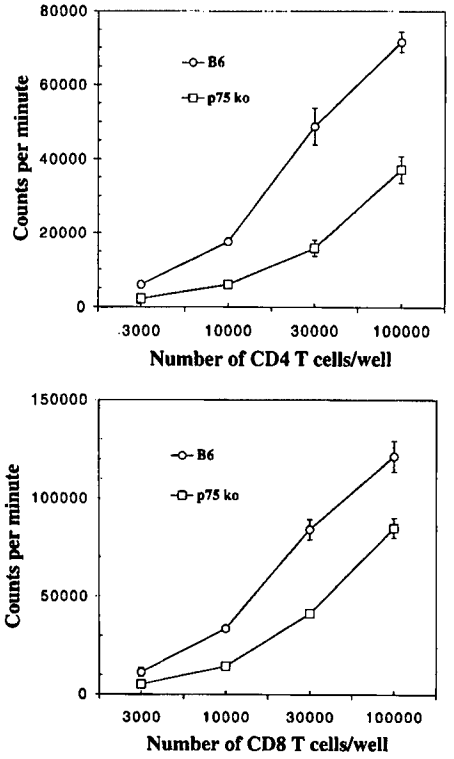

FIGURE 6. p75 is required for the optimal proliferation of anti-TCR-stimulated CD4 and CD8 T cells. The indicated number of purified CD4 and CD8 T cells from B6 and B6-p75$^{-/-}$ mice were cultured in wells coated with 10 μg/ml of 2C11 in I-medium containing 20 U/ml of IL-2. One μCi of [^{3}H]thymidine was added to each culture during the last 6 h of a 48-h culture period. The error bars represent the SDs of triplicate cultures.

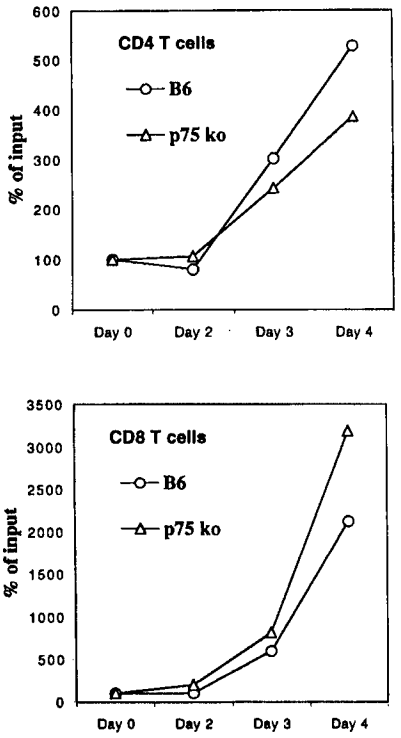

FIGURE 7. Higher recovery of anti-TCR-stimulated p75$^{-/-}$ CD8 T cells after 4 days of culture. Spleen cells (1×10^5) from B6 and B6-p75$^{-/-}$ mice were cultured in wells coated with 10 μg/ml of 2C11 in I-medium containing 20 U/ml of IL-2. The percentage of CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells recovered after 2, 3, or 4 days of culture was determined by FACS analysis. The total number of CD4 or CD8 cells recovered from each culture at these times was determined and expressed as a percentage of the starting population.

Activated CD8 T cells lacking the p55 receptor are not killed by TNF plus cycloheximide (Fig. 5); this observation suggests that TNF-induced cell death in activated CD8 T cells operates through the p55 receptor. 2) Activated B6 CD8 T cells, which express Fas, can be killed very efficiently by FasL$^+$ fibroblasts (Fig. 4). This observation indicates that the Fas/FasL-mediated death pathway is not defective in activated CD8 T cells. 3) Activated p55$^{-/-}$ CD8 T cells, which express the same level of the p75 receptor as activated B6 CD8 T cells, are not killed by TNF plus cycloheximide (Fig. 5), suggesting that TNF-dependent death signals are not transmitted through the p75 receptor.

Instead of mediating TNF-induced cell death, we found that the p75 receptor is required for efficient Fas/FasL-mediated killing of activated CD8 T cells (Fig. 4). Together with the finding that the p75 receptor augments TNF/p55-mediated killing, our data are more consistent with the following alternative explanations for the resistance of activated p75$^{-/-}$ CD8 T cells to TCR-induced cell death: 1) activated p75$^{-/-}$ CD8 T cells are highly resistant to Fas/FasL-mediated killing, and 2) the reduced sensitivity of these cells to TNF/p55-mediated killing.

What are the potential explanations for the relative resistance of activated p75$^{-/-}$ CD8 T cells to Fas/FasL-mediated death pathway? Previous studies have shown that Fas-mediated killing as a result of TCR stimulation is dependent on the induction of FasL expression after TCR stimulation (31–33). However, we found that FasL induction is not defective in TCR-stimulated p75$^{-/-}$ CD8 T cells (Fig. 3). Mature T cells have been shown to express high levels of Fas-associated death domain-like IL-1-converting enzyme-inhibitory proteins (FLIPs) that block death receptor-induced cell death (41). Significantly, T cells down-regulate FLIP when they are activated in vitro, and this down-regulation correlated with the sensitivity of the activated T cells to Fas/FasL-mediated cell death (42–44). Retrovirus-mediated expression of cellular FLIP (c-FLIP) blocks Fas-induced apoptosis of activated T lymphocytes, but does not affect cell death resulting from cytokine withdrawal (44). It remains to be determined whether the p75 receptor complex may recruit and sequester c-FLIP away from the Fas receptor complex and in this way interferes with Fas/FasL-mediated killing. The p75 receptor complex can also recruit cellular inhibitors of apoptosis proteins (c-IAPs) (45), which contribute to antiapoptotic signals (46, 47). Therefore, another potential mechanism for the regulation of Ag-induced cell death by the p75 receptor is through the sequestration of proteins such as c-IAPs away from the Fas receptor complex.

It is interesting to note that Fas/FasL-mediated killing of activated CD4 T cells is not regulated by the p75 receptor. A potential explanation for the independence on the p75 receptor of activated CD4 T cells to Fas-mediated killing is that inhibitors of Fas-mediated killing such as c-FLIP and/or c-IAPs may be present at relatively low levels in activated p75$^{-/-}$ CD4 T cells, and the sequestration of these factors by the p75 receptor complex may not be required for Fas-mediated killing of activated CD4 T cells. This possibility is being investigated.

The lpr *and* gld *phenotypes are affected by p55$^{-/-}$ and TNF$^{-/-}$ mutations*

The *lpr* phenotype is accelerated in p55$^{-/-}$ *lpr/lpr* mice (48). More recently, it was shown that TNF$^{-/-}$ *gld/gld* mice have a much less

severe *gld* phenotype (49). The lymphoproliferative disorder in *lpr* and the *gld* mice is due to the accumulation of the $CD4^-CD8^-\alpha\beta$ TCR^+ $B220^+$ (double-negative) T cell population in the peripheral lymphoid organs of these mice (5). It is conceivable that in $p55^{-/-}$ *lpr/lpr* mice, there is a lack of signaling through p55, but signaling through the p75 receptor can still occur. We speculate that signaling through the p75 receptor may promote the proliferation of double-negative cells and exacerbates the *lpr* phenotype. The requirement for the p75 receptor for optimal proliferative responses is most likely due to the activation of NF-κB-mediated signaling pathways (23, 24), which has been shown to antagonize TNF-mediated apoptosis (50–52). By contrast, in $TNF^{-/-}$ *gld/gld* mice, signaling through the p75 receptor cannot occur and proliferative signals offered by the p75 receptor are lacking for these cells, and this may account for the less severe *gld* phenotype.

The studies in the *lpr* and *gld* mice and our present work illustrate that Fas and TNF signaling pathways are intimately related. We have demonstrated that signaling through TNFR1 and the Fas receptor can be regulated by TNFR2 signals. Delineation of the nature of the cross-talk between distinct receptors of the TNFR family will undoubtedly lead to novel insights regarding the multiple functions of these receptors and the molecular basis for the multifaceted manifestations of the lymphoproliferative and autoimmune disorders associated with the *gld* and *lpr* mutations.

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Poor Binding of a HER-2/neu Epitope (GP2) to HLA-A2.1 Is due to a Lack of Interactions with the Center of the Peptide*

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Class I major histocompatibility complex (MHC) molecules bind short peptides derived from proteins synthesized within the cell. These complexes of peptide and class I MHC (pMHC) are transported from the endoplasmic reticulum to the cell surface. If a clonotypic T cell receptor expressed on a circulating T cell binds to the pMHC complex, the cell presenting the pMHC is killed. In this manner, some tumor cells expressing aberrant proteins are recognized and removed by the immune system. However, not all tumors are recognized efficiently. One reason hypothesized for poor T cell recognition of tumor-associated peptides is poor binding of those peptides to class I MHC molecules. Many peptides, derived from the proto-oncogene HER-2/neu have been shown to be recognized by cytotoxic T cells derived from HLA-A2$^+$ patients with breast cancer and other adenocarcinomas. Seven of these peptides were found to bind with intermediate to poor affinity. In particular, GP2 (HER-2/neu residues 654–662) binds very poorly even though it is predicted to bind well based upon the presence of the correct HLA-A2.1 peptide-binding motif. Altering the anchor residues to those most favored by HLA-A2.1 did not significantly improve binding affinity. The crystallographic structure shows that unlike other class I-peptide structures, the center of the peptide does not assume one specific conformation and does not make stabilizing contacts with the peptide-binding cleft.

Class I major histocompatibility complex (MHC)[1] proteins bind short peptides (9–11 amino acids) derived from cytosolically degraded proteins. These peptides are transported into the endoplasmic reticulum and bind to newly formed class I molecules. Peptide binding appears to be the final step in assembly of the complex (1). Following peptide binding, the complexes are transported to the plasma membrane. At the plasma membrane, clonotypic T cell receptors on the surface of circulating cytotoxic T lymphocytes (CTL) may recognize the peptide-MHC complex (pMHC). If the pMHC is recognized by the T cell receptor, the T cell is activated and the cell presenting the pMHC is killed. A normal cell will have a large assortment of pMHC on the cell surface that are not recognized by CTL. However, viral or mutated self-proteins are degraded by these same mechanisms, and many of the resulting pMHC are recognized by CTL. In this manner, virus-infected or mutated cells are targeted for lysis by cytotoxic T cells (reviewed in Ref. 2). Self-proteins that are expressed in abnormally high amounts or in abnormal cell types may also be targets for CTL (3).

Class I MHC molecules bind many peptides with diverse sequences and high affinity (4). To bind all these peptides, the class I protein primarily interacts with the invariant portions of the peptides, the N and C termini (5). Class I MHC also uses a subset of amino acid side chains within the peptide termed "anchors" to generate significant binding (6). These peptide anchor residues bind within "specificity pockets" that are primarily formed by the polymorphic residues within the peptide-binding cleft of the MHC molecule (7). Peptides that bind with high affinity to a given allotype are typically found to have one of a few preferred amino acids at each anchor position. The corresponding hypothesis is that peptides that do not have those preferred amino acids at the anchor positions will not bind well. The combination of amino acids that may bind at the anchor positions is known as the peptide-binding motif (8). These motifs have proven to be extremely valuable in predicting peptides that will bind to class I MHC. Other residues within the peptide besides the anchors may be used to generate increased binding affinity (9–11).

Interestingly, many peptides that appear to have the correct peptide-binding motif still bind poorly. Substituting the anchor residues of poor binding peptides with those that are most preferred by the allotype can generate high affinity binding. (10, 12). Some of these altered peptide ligands (APL) are even effective therapeutics (13). We show here that there are also peptides for which altering the anchor residues does not significantly increase binding affinity. It is not clear from the previously available data in the literature why these peptides bind poorly.

HER-2/neu (c-erb-2) encodes a receptor tyrosine kinase with homology to the epidermal growth factor receptor. Overexpression of HER-2/neu in many adenocarcinomas, including breast and ovarian tumors, correlates with a poor prognosis for remission and recovery (14). Tumor infiltrating lymphocytes have been found in cancer patients that overexpress HER-2/neu, and these tumor infiltrating lymphocytes are able to recognize and lyse the solid tumor (3, 15, 16), but these CTL do not eliminate the tumor. It has also been shown that several peptide epitopes derived from the gene product of HER-2/neu are presented by class I MHC molecules to circulating CTL. As with many other

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The atomic coordinates and structure factors (code 1QR1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

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[1] The abbreviations used are: MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; pMHC, peptide-MHC complex(es); APL, altered peptide ligand(s); HPLC, high pressure liquid chromatography; BFA, brefeldin A; A2, HLA-A2.1.

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tumor-associated antigens, most of these peptides bind poorly to HLA-A2.1 (A2). There are many potential reasons for the lack of immune removal of tumors including the down-regulation of class I MHC or down-regulation of the protein from which the peptide is derived. It has also been proposed that one reason for poor recognition by CTL is weak binding of the immunogenic peptides to class I MHC (3).

Here we show that HER-2/neu-derived peptides, identified in the literature as recognized by CTL, bind with a range of affinities, but all are lower affinity than two index peptides of high affinity. One peptide was chosen for further study. This peptide, GP2 (IISAVVGIL), binds very poorly to A2 but has anchor residues that are present in high affinity peptides (Ile at position 2 and Leu at position 9). Its inherently poor affinity is not significantly increased by substitution of its anchor residues. To understand why this peptide binds poorly, the crystallographic structure of the A2-GP2 complex was determined. Unlike all previously determined peptide-class I MHC (pMHC) structures, there is a large region of unresolved electron density in the center of the peptide. We interpret this to mean that the peptide assumes more than one conformation within the peptide-binding cleft. We hypothesize that the observed poor binding is due to the lack of important secondary interactions within the center of the peptide.

EXPERIMENTAL PROCEDURES

Preparation of HLA-A2.1-Peptide Complexes—Residues 1–275 of HLA-A2.1 (A2) and residues 1–99 of human β_2-microglobulin were expressed in *Escherichia coli*, produced as inclusion bodies, purified, and folded as described previously (17). Briefly, peptide, solubilized β_2-microglobulin and solubilized A2 heavy chain were rapidly diluted into folding buffer (10 mM Tris, pH 8.0, 0.4 M L-Arg, 10 mM reduced glutathione, 1 mM oxidized glutathione, and protease inhibitors) at molar ratios of 10:5:1, respectively. The final protein concentration was kept below 50 μg ml^{-1}. The solution was incubated at 10 °C for 36–48 h and then concentrated (Amicon) and purified by HPLC gel filtration (Phenomenex, BioSep-SEC-S2000).

Synthetic Peptides—All peptides were synthesized by the Peptide Synthesis Facility at the University of North Carolina at Chapel Hill. The peptides were purified to greater than 95% purity by reversed-phase HPLC and identity confirmed by matrix-assisted laser desorption ionization-time-of-flight spectroscopy. Peptides were dissolved in 100% dimethyl sulfoxide at 20 mg ml^{-1} by weight. The final peptide concentration was determined by amino acid analysis (Protein Chemistry Laboratory, Department of Chemistry, University of North Carolina, Chapel Hill). The list of peptides and references for immunogenicity are given in Table I.

Determination of Thermal Stability—Purified A2-peptide complexes were exchanged into a 10 mM KH_2/K_2HPO_4 buffer, pH 7.5, and adjusted to a final protein concentration of 4–12 μM. The change in CD signal at 218 nm was measured as a function of temperature from 4 to 95 °C on an AVIV 62-DS spectropolarimeter (Aviv Associates Inc, Lakewood, NJ). The final melting curve was the average of at least three experiments for each A2-peptide complex. T_m values were calculated as the temperature at which 50% of the complexes are denatured using a two-state denaturation model (12).

Cell Surface Stabilization Assay—Cell surface stabilization of A2 was performed as described previously (11). Briefly, 2.5 × 10^5 T2 cells (ATCC CRL-1992) were incubated overnight in AIM V serum-free medium (Life Technologies, Inc.) at 37 °C, 5% CO_2 in the presence of GP2 or APL, at concentrations varying from 50 to 0.05 μM. Cells were then stained with the monoclonal antibody BB7.2 (18) specific for A2, followed by fluorescein isothiocyanate-labeled (1:50) goat anti-mouse IgG antibody (Southern Biotechnology Associates). Cells were analyzed by flow cytometry (FACScan, Becton Dickinson), and the mean channel fluorescence was determined using the CYCLOPS software package (Cytomation, Fort Collins, CO). All data are normalized as the percentage of the mean channel fluorescence for the calreticulin-signal-sequence peptide, ML, bound to A2 at 50 μM. Binding by the A2-specific antibody, BB7.2, was not dependent on the peptide bound because W6/32, an antibody that binds to an epitope between the α3 domain and β_2-microglobulin, gave similar results (data not shown).

Cell Surface Half-life Assay—The determination of cell surface half-lives ($T_{1/2}$) of A2-peptide complexes was performed as described previously (11). Briefly, 2.5 × 10^6 T2 cells were incubated overnight in AIM V serum-free medium at 37 °C, 5% CO_2 in the presence of 50 μM peptide. To block the egress of new A2 molecules to the surface, cells were incubated at 37 °C, 5% CO_2 in RPMI 1640, 15% fetal calf serum and 10 μg ml^{-1} brefeldin A (BFA, Sigma). This concentration of BFA is toxic to the cells. Therefore, after 1 h the cells were then transferred to RPMI 1640, 15% fetal calf serum, and 0.5 μg ml^{-1} BFA. At the indicated time points, 2.5 × 10^5 cells were removed, incubated with BB7.2, and analyzed by flow cytometry as described above for cell surface stabilization assay. Each time point is evaluated as mean fluorescence with peptide minus mean fluorescence without peptide and normalized to the maximal level of fluorescence (at time zero) for each peptide.

TABLE I

Summary of binding data of HER-2/neu-derived peptides to A2

Residues substituted with respect to wild-type peptide are shown in boldface type. T_m is the temperature (°C) at which 50% of the protein is denatured as measured by circular dichroism. K_r is the relative binding constant as determined by the T2 cell surface assembly assay. K_r is defined as the concentration of peptide in μM that yields 50% mean channel fluorescence as compared to the maximum fluorescence of the control peptide (ML) at 50 μM. The K_r value for ML is the concentration that yields 50% mean channel fluorescence. $T_{1/2}$ is the half-life of peptide-A2 complexes (in hours) as determined by the T2 cell surface stability assay. ND, not determined. DNF, did not fold *in vitro*. The error in the T_m is the sum of the machine and curve fit errors. It is typically about 1 °C. >50 means that the concentration to yield 50% of ML fluorescence is greater than 50 μM.

Peptide	Sequence	T_m	K_r	$T_{1/2}$
GP2 (16)	IISAVVGIL	36.4	>50	0.35
S1 (38)	SIISAVVGI	44.2	13.2	4.05
L10 (38)	IISAVVGILL	41.3	>50	1.58
E74 (15)	DVRLVHRDL	DNF	>50	DNB
E75 (15)	KIFGSLAFL	45.1	14.4	8.57
F56 (39)	YISAWPDSL	34.8	>50	DNB
C84 (39)	ELVSEFSRV	37.5	>50	0.30
L9V	IISAVVGI**V**	38.8	>50	0.69
I2L	I**L**SAVVGIL	42.2	22.9	1.76
I2L/L9V	I**L**SAVVGI**V**	42.5	10.0	2.48
ML (30)	MLLSVPLLL	52.5	1.8	19.53
RT (31)	ILKEPVHGV	50.0	7.7	9.69
MelA (36)	EAAGIGILTV	40.9	47.2	0.44
MelA-A2L	E**L**AGIGILTV	50.0	1.6	9.98

Crystallization, Data Collection and Processing—Crystals were grown by hanging drop vapor diffusion as described previously (19). Crystallographic data of A2-GP2 were collected on a single crystal at the National Synchrotron Light Source, Brookhaven National Labs, beamline X-12B at −170 °C (Oxford Cryosystems). Evaluation of the diffraction pattern with DENZO autoindexing function showed the space group to be triclinic P1. 180 degrees of data were collected at a distance of 90 mm from the Quantum 4 CCD detector (ADSC Poway, CA) with one-degree oscillations and 3 min of exposure time/frame. Data were processed with DENZO, and intensities were scaled with SCALEPACK (20). Data statistics are shown in Table II.

Structure Determination and Refinement—The A2-GP2 structure was determined by molecular replacement using AMoRe (21) within the CCP4 program suite (22). The A2-hepatitis peptide complex (PDB accession code 1HHH) was used as the search model (23). Because the domains tend to vary in their relative orientations with respect to one another in different crystal forms, the search model was divided into three pieces, the peptide-binding superdomain ($\alpha_1\alpha_2$), the α_3 domain, and β_2-microglobulin. Initial rounds of positional refinement used X-PLOR from 8–2.4 Å resolution data. Later rounds were performed with Refmac using all data from 30.0 to 2.4 Å. Final rounds of refinement used torsional dynamics with CNS (24–26) with all data. Electron density maps were generated using DM and functions for 2-fold non-crystallographic averaging, histogram matching, and solvent flattening. Manual intervention was performed using O (27). 103 water molecules were added to the structure using the program ARP (28) combined with Refmac and confirmed by visual inspection of the electron density maps. The refinement statistics are listed in Table II.

RESULTS

HER-2/neu-derived Peptides Bind Poorly to A2—We began these studies to assess the correlation of immunological activity with peptide binding affinity to HLA-A2.1 (A2). Thermal stability of class I MHC-peptide complexes, as measured by

TABLE II
Summary of crystallographic data

The crystallographic structure of A2-GP2 was determined by molecular replacement using the A2-hepatitis B 10-mer (Protein Data Bank code 1HHH) as the search model. The structure was refined by a combination of X-PLOR and Refmac. Individual Bs were refined in the penultimate cycle followed by the addition of waters.

Data statistics	
Space group	P1
Cell Dimensions	a = 50.34 Å b = 63.61 Å c = 75.14 Å α = 81.98 ° β = 76.25 ° γ = 77.83 °
Molecules/Asymmetric Unit	2
Resolution	30–2.4 Å
R_{merge} (%)[a]	9.3 (23.3)[b]
$<I/\sigma>$	7.80 (3.46)
Unique reflections	34,962
Total reflections	66,839
Completeness (%)	98.2 (97.6)
Refinement	
Resolution	30–2.4 Å
R_{free} (%) (number of reflections)[c]	28.4 (1,714)
R_{work} (%) (number of reflections)[c]	24.2 (31,969)
Rs fit[d]	83.8%
No. of non-hydrogen atoms	6,292
No. of waters	103
Error[e]	0.26 Å
Average B factor	16.8 Å^2
R.M.S. deviations from ideality	
Bonds	0.009 Å
Angles	1.468 °
Residues in Ramachandran plot	
Most favored	91.6%
Additional allowed	8.1%
Generously allowed	0.3%
Disallowed	0.0%

[a] $R_{merge} = \Sigma_{hkl} \Sigma_i |I_i - <I>| / \Sigma_{hkl} \Sigma_i I_i$, where I_i is the observed intensity and $<I>$ is the average intensity of multiple observations of symmetry related reflections.

[b] Number in parenthesis refers to the highest resolution shell (2.44–2.40) for A2-GP2 unless otherwise stated.

[c] $R = \Sigma_{hkl} | |F_{obs}| - k |F_{cal}| | / \Sigma_{hkl} |F_{obs}|$, where R_{free} is calculated for a randomly chosen 5% of reflections and R_{work} is calculated for the remaining 95% of reflections used for structure refinement.

[d] Rs fit is the average real space fit of all atoms on an electron density map from DM with 2-fold noncrystallographic averaging, histogram matching, and solvent flattening.

[e] Error is the mean estimate of the coordinate error based on maximum likelihood methods (Refmac).

circular dichroism, have been shown to correlate with the free energy of peptide binding to class I MHC (29). Therefore, the thermal stabilities of recombinant A2 complexes folded *in vitro* with seven HER-2/neu peptides identified as important epitopes for breast cancer immunotherapy in the literature (GP2, S1, L10, E74, E75, F56, and C84; see Table I for sequences) were determined. As can be seen from Fig. 1*A*, complexes formed with GP2, F56, and C84 have extremely low melting temperatures. Complexes formed with S1, L10, and E75 (summarized in Table I) have higher melting temperatures. E74 bound so poorly as to be undetectable in any of our assays (data not shown). A cell surface binding assay (Fig. 1*B*) using T2 cells with exogenously added peptide confirms the results found by the circular dichroism experiments. Two peptides, one hydrophobic and one hydrophilic, were chosen as representative "high affinity" binders. ML is derived from the signal sequence of calreticulin (30), and RT is derived from HIV-1 reverse transcriptase (31). The thermal stability (T_m) and the relative binding constant (K_r) determined by the T2 assay correlate well (91.3% correlation coefficient). This suggests that K_r is proportional to K_D, because, as stated above, the T_m has previously been shown to be proportional to the K_D (29).

Adding BFA to the cell surface stability assay allows us to measure the amount of time a peptide-MHC complex stays on the surface of cells. As can be seen in Fig. 1*C*, the GP2 peptide has an extremely short half-life of ~21 min at 37 °C (Table I). Some of the other HER-2/neu-derived peptides have longer half-lives, but none are as long as peptides such as ML or RT (Fig. 1*C* and Table I). We could not detect binding of the E74 peptide in any of our assays. The fact that CTL activity toward E74 can be seen and we cannot measure binding is probably a function of the extreme sensitivity of CTL. Only ~100 class I-peptide complexes/cell are required to trigger an activated T cell (32).

Anchor Substitutions Do Not Significantly Improve Binding of GP2—Substitutions at peptide anchor positions have been shown to greatly increase the thermal stability of an influenza matrix peptide (12). In the present work, we chose one of the three poor binding peptides, GP2, to study the anchor substitutions of HER-2/neu-derived peptides. The GP2 anchors (Ile at position 2 and Leu at position 9 of the peptide) are found in peptides that bind with high affinity to A2, but these anchors are not optimal (8). Therefore, optimized APL based on GP2 were synthesized that replaced the Ile at position 2 with Leu (I2L) or the Leu at position 9 with Val (L9V). As can be seen in Fig. 1*D*, these substitutions did increase the thermal stability (~2–6 °C) but not to the degree that was seen for similar substitutions in the influenza matrix peptide (~7–9 °C) (12) or for a variant of a melanoma peptide (MelA and MelA-A2L, ~9 °C). The cell surface stability assay using T2 cells supports the CD data that we have measured (Fig. 1*E*). The half-lives of the APL complexes on the cell surface are increased with respect to GP2 (Fig. 1*F*). However, they are not close to the time constants seen for the positive control, high affinity binders ML or RT.

Crystallographic Structure of A2-GP2—To understand why GP2 binds poorly to A2 and why the anchor substitutions do not significantly increase the stability, we determined the crystallographic structure of A2-GP2. The molecular replacement solution was unambiguous with a correlation coefficient of ~73% after rigid body fitting. The model was refined in X-PLOR (33). During refinement, the peptide was omitted to reduce the potential for model bias. Density modification was performed with DM (22) using the X-PLOR output coordinates to generate unbiased averaged electron density maps of the peptide and to fit the structure of A2. Unlike all of the pMHC structures that we have determined to date, the entire length of the main chain of the peptide was not visible in the density modified electron density maps at this stage. After 10 cycles of model building with O (27) and computational refinement with X-PLOR and Refmac and finally with CNS, the refinement converged to the statistics shown in Table II. In general, the maps are clear and unambiguous. The entire A2 molecule is well resolved and fits the density well as evidenced by an average real space correlation coefficient of 83.8%. The positions of the termini of the GP2 peptide are also unambiguous and never altered through the course of refinement. However, unlike all reported pMHC structures, the center of the peptide never became clear in the density (Fig. 2). In addition, standard $2F_o - F_c$ maps, simulated annealing omit maps, unaveraged omit maps, and composite omit maps failed to show density for the center of the peptide. In particular, the orientation of residue 6 (Val) is completely uninterpretable, and the positions of residues 5 and 7 (Val and Gly, respectively) are not well defined.

DISCUSSION

One hypothesis used to explain why tumors are not recognized and eliminated by the immune system is that potentially

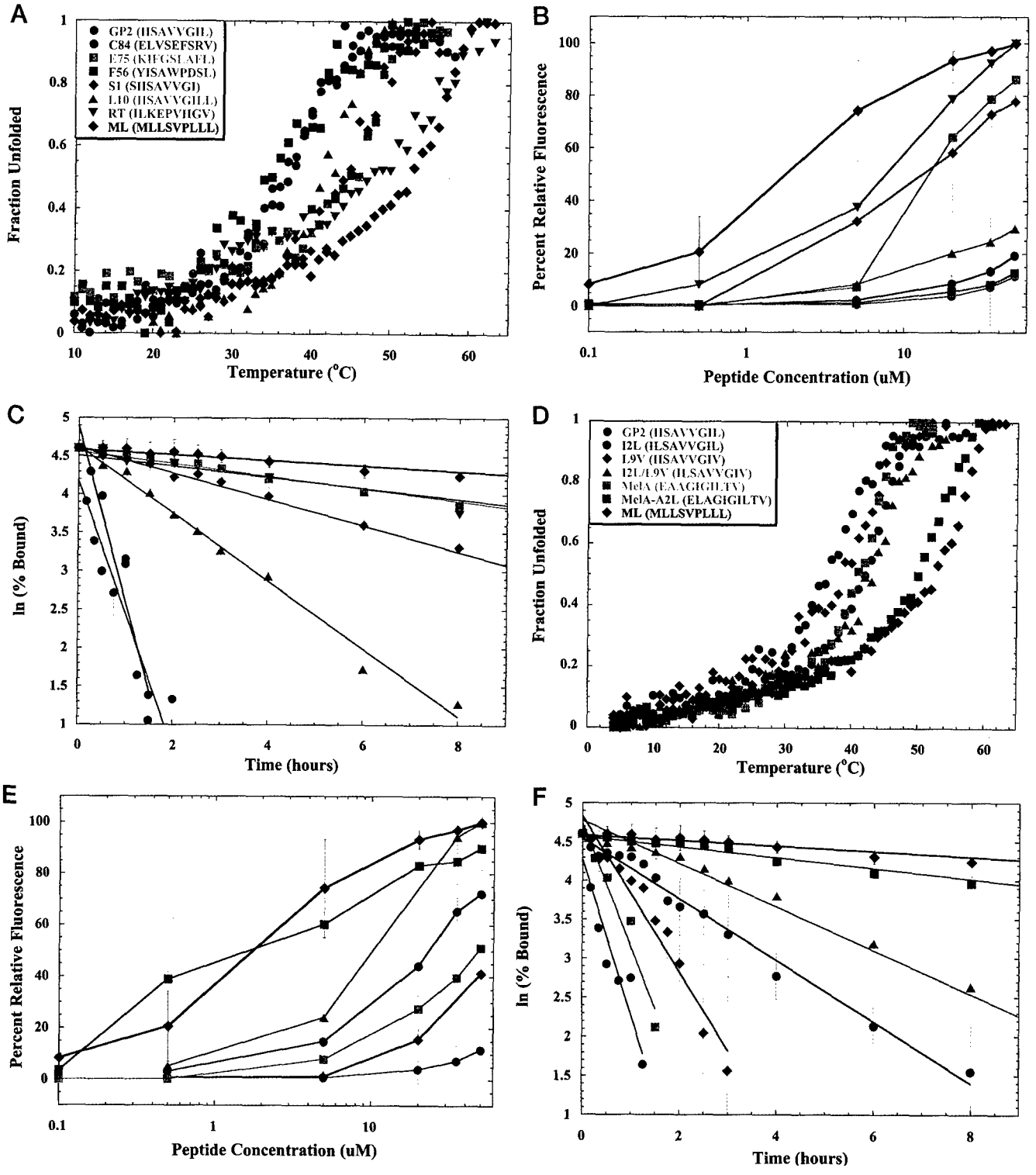

FIG. 1. **HER-2/neu-derived peptides bind poorly to A2, and anchor substitutions do not increase the stability of GP2-derived APL.** The symbols and colors shown in *A* are also those used in *B* and *C*. Likewise, the symbols and colors shown in *D* are also those used in *E* and *F*. *A*, thermal stability of A2-peptide complexes as measured by CD. 4–12 μM protein was denatured by heat in a circular dichroism spectropolarimeter. The change in CD signal at 218 nm is an indication of the loss of secondary structure within the protein. Each curve is the average of three independent experiments. The error in the T_m is the sum of the curve fit error and the Peltier temperature controller error and is ~1 °C. *B*, cell surface measurements confirm relative affinities measured by circular dichroism. T2 cells were incubated with the indicated concentrations of peptide and the amount of cell surface A2 measured by flow cytometry using an A2-specific monoclonal antibody BB7.2. *C*, cell surface half-lives of A2-peptide complexes were determined by treating the peptide-pulsed cells (as in *B*) with BFA to halt vesicular transport. Aliquots of cells were removed at the indicated times and the remaining A2 on the cells determined by incubating with BB7.2. *D*, CD experiments show that anchor substitutions of GP2 do not greatly increase the stability. The best peptide is the double substitution I2L/L9V, but even it is deficient compared with ML. *E*, T2 cell surface stabilization confirms the CD data. *F*, the cell surface half-lives are moderately increased compared with GP2.

immunologically reactive peptides do not bind well to class I MHC molecules. If the peptides dissociate from class I MHC molecules too quickly, the cells presenting the peptides do not have a sufficient concentration of the specific pMHC at the surface of the cell to be recognized by circulating T cells. We examined binding of a selection of known immunologically recognized

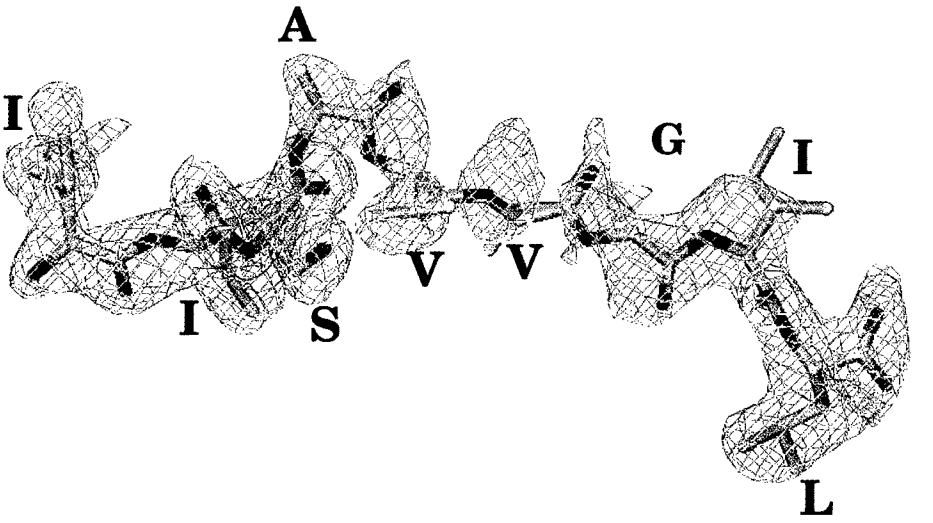

FIG. 2. **The center of the GP2 peptide is disordered.** The averaged omit electron density map of the GP2 peptide with a cover radius of 1.5 Å. The map was calculated using modified phases from DM.

peptide ligands from the tyrosine kinase family member HER-2/neu. Despite the presence of CTL that recognize these peptides bound to A2, the tumors are not eliminated. These HER-2/neu peptides displayed a spectrum of binding affinities, but all were lower than the level observed for high affinity binders, such as ML or RT. Of particular interest to the immunology of tumor recognition was the clustering of many of these peptides in the "low affinity" category. Remarkably, all of these peptides, (GP2, C84, and F56) have good anchor residues for A2.

There are two primary reasons to examine this phenomena in detail. The first is to understand how class I MHC binds peptides. There is a great deal known about how class I MHC binds many peptides with great sequence diversity, but there is very little information about how the protein binds any particular peptide well or poorly. There are now many examples of crystal structures of high affinity peptides bound to class I MHC. GP2 is a perfect example of a poor binding peptide and as such offers the first opportunity to understand poor binding. The second reason to examine GP2 is that poor affinity peptides are potentially better targets for immunotherapy. The rationale for this has to do with T cell education. T cells are selected for survival by two mechanisms (positive and negative selection) in the thymus (35). If a self-peptide binds to class I MHC with high affinity, there is a larger concentration of pMHC in the thymus and thus a greater chance that T cells would be able to recognize the complex well. Presumably, this set of T cells would be deleted from the T cell receptor repertoire, and they would not be in the periphery. If the self-peptide binds with poor affinity, the concentration of that peptide-MHC molecule in the thymus may be too low for recognition during the selection process. Therefore, there is a greater probability of finding these T cells in the periphery.

A complex of A2 with GP2 bound has poor thermal stability (T_m, 36.4 °C) and a very short cell surface half-life (~21 min). Many laboratories including ours have improved the binding affinity of peptides by changing the anchor residues to those most preferred by A2 (13, 36). As an example, we show that a small change in the anchor position of the melanoma peptide MelA results in a peptide with much greater binding affinity. However, trials with substitutions of GP2 at the anchor positions showed that the affinity was not significantly improved (Table I). Our goal is to be able to design APL for cancer immunotherapy. To be able to do this in a reasonable fashion, we needed to determine the crystallographic structure of A2-GP2 and determine why this peptide binds poorly.

The crystallographic structure shows uninterpretable electron density within the center of the peptide. Our interpretation of these data is that the peptide does not assume one unique conformation in the center as has been seen for all other single peptide-MHC structures to date (reviewed in Ref. 4). Interestingly, this is analogous to the situation found in the crystal structure of a class I MHC complex that contained a mixture of many different peptides (37). These data suggest that anchor substitutions do not significantly increase the affinity of GP2 because they do not address the fundamental problem that the peptide has in binding. The center does not make stabilizing contacts with the binding cleft of the class I MHC molecule.

This result begs another important immunological question. Does the flexibility in the center of the peptide increase or decrease immunogenicity? On the one hand, the flexibility decreases the already small concentration of a specific molecular surface that can interact with the T cell receptor on a circulating T cell. On the other hand, multiple peptide conformations generate more molecular surfaces that can be potentially recognized by circulating T cells. Perhaps in the context of a peptide that binds well, increased flexibility is more immunogenic, but in the context of a poor binding peptide increased flexibility does not increase immunogenicity because of the reduced concentration effect.

There is increasing interest in using peptides that bind to class I MHC for immunotherapy. As is the case of vaccination used to prevent viral infection, the potential therapeutic value is significant. As more antigens are discovered that are recognized by CTL and yet bind poorly to class I MHC molecules, the rules that predict binding affinity will be more critical. The phenomena observed here for GP2 certainly applies to other poor binding peptides whose binding affinity is not increased by altering the anchor residues. Increased affinity can be obtained for many of these peptides, but a full understanding of how peptides bind to class I MHC is still needed. By examining the binding of GP2 at the atomic level, we have made another step toward understanding peptide binding well enough to make predictions that will increase peptide affinity and minimize immunological consequences.

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Class I MHC Is Stabilized Against Thermal Denaturation by Physiological Concentrations of NaCl

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Class I MHC Is Stabilized Against Thermal Denaturation by Physiological Concentrations of NaCl†

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ABSTRACT: Class I MHC molecules are ternary complexes composed of an allotype specific heavy chain, a noncovalently associated protein β_2-microglobulin (β_2m), and a peptide. The complexes are assembled in the endoplasmic reticulum by a complex series of chaperones and peptide-loading mechanisms. In the absence of β_2m or peptide, very little class I heavy chain is transported to the surface of the cell. Complexes that do not contain all three parts of the protein are not made productively in vivo and not at all in vitro. The ability of the complex to withstand thermal denaturation in vitro has been shown to be related to the binding affinity of the peptide. Paradoxically, some low-affinity peptide complexes denature at or below human basal body temperatures in vitro but are effective biological agents in vivo. Here we show that these complexes are stabilized against thermal denaturation by physiological cosolvents and maximally stabilized by 150 mM NaCl. While the degree of stabilization by 150 mM NaCl is greatest for low-affinity peptide/MHC complexes, the mechanism of stabilization is independent of peptide sequence. This effect is hypothesized to occur by multiple mechanisms including increasing the affinity of β_2m for the complex and charge screening.

Class I molecules are ternary complexes that are expressed on the plasma membrane of nearly all cells in the body (*1*). These molecules are composed of a 44 kDa polymorphic heavy chain, a noncovalently associated light chain (~11 kDa) called β_2-microglobulin (β_2m),[1] and a small peptide (*2*). In vitro studies typically only use the extracellular portion of the heavy chain (*2*−*4*) which contains the peptide-binding superdomain and an immunoglobulin domain (*5*). Both the peptide-binding superdomain and the immunoglobulin domain make extensive contacts with β_2m (*6*).

Class I MHC molecules bind small peptides (8−11 amino acids) and present them to circulating T cells at the plasma membrane. In so doing, the class I MHC molecules serve to signal to T cells the identity of the proteins being expressed within the cell. The peptides that class I MHC bind are endogenously derived from host or intracellular pathogens. They are processed by the proteasome in the cytosol and are transported into the endoplasmic reticulum by specific transporters (*7*). The peptides are actively loaded onto class I MHC molecules within the endoplasmic reticulum before export to the cell surface (*1*). These peptide/MHC (pMHC) complexes are recognized by clonotypic T cells via their T cell receptors (TCR). Upon proper binding of TCR with pMHC, the T cells lyse the cell. A properly functioning cell will present unmutated self-peptides with class I MHC. These cells are typically untouched by the immune system, because either self-reactive T cells are eliminated during T cell development or they are functionally inactivated in the periphery. Cells that are infected by a virus or transformed to a cancerous state by DNA mutation present peptides that have not been seen by the immune system. Thus, this signal of the state of the cell prevents further viral or tumor propagation by selectively destroying aberrant cells [reviewed in (*8*)].

Class I MHC molecules bind many different peptides primarily through the invariant peptidic termini. These termini dock in pockets composed of conserved amino acids found within a large cleft in a superdomain formed by the α1 and α2 domains of the heavy chain (*5*). Altering the chemical nature of the peptidic termini drastically reduces

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[1] Abbreviations: CTL, cytotoxic T cell; TCR, T cell receptor; MHC, major histocompatibility complex; pMHC, peptide/MHC complex; A2, human class I MHC HLA-A2.1; D^b, murine class I MHC molecule H-2D^b; K^b, murine class I MHC molecule H-2K^b; β_2m, β_2-microglobulin (h or m prefix refers to human or murine protein); HEL, hen egg white lysozyme; CD, circular dichroism spectropolarimetry; T_m, temperature at which 50% protein is denatured.

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the stability of the class I MHC complex as measured by thermostability experiments (*9*). Sequencing of pooled peptides eluted from class I (*10*) and determination of individual sequences (*11, 12*) showed that class I MHC restricts the use of amino acids at specific positions within the antigenic peptide. These positions within the peptides are termed anchors; a combination of anchors constitutes a peptide-binding motif (*5, 13*−*15*). The anchors are bound by the polymorphic residues within the peptide-binding superdomain of class I MHC. Because the polymorphic nature of the cleft forms the basis for the amino acids selected within the peptide, the motifs are specific for each class I allotype. These anchor residues may (*9, 16*) or may not (*17*) be used to generate significant binding free energy. It is unclear how many peptides may really be able to bind to each class I MHC. Estimates of the numbers of peptides bound to a class I MHC allotype on the cell surface have been in excess of 10^4 based on mass spectrometry (*18*), but are surely underestimated because of sampling error due to MHC preparation and vaporization for mass spectrometry.

Immunotherapies for cancer or viral infection that utilize class I molecules and peptides have been proposed (*19*). For this therapy to be successful, some critical quantity of class I molecules have to present antigenic peptide for a significant duration to signal the immune system (*20*). Understanding what makes a particular peptide bind well and another bind poorly is of critical importance to this work. Unfortunately, little is known about the mechanism of peptide binding beyond qualitative descriptions.

Poor peptide binding to class I has been postulated to be the cause for inefficient immunogenicity against tumor antigens in vivo (*21, 22*). Clearly, if a peptide does not bind well, the pMHC complex cannot be detected by circulating T cells. We examined binding of a group of peptides that are known to be recognized by cytotoxic T cells (CTL) derived from the tyrosine kinase HER-2/neu using circular dichroism (CD) spectropolarimetry and flow cytometry. Previous experiments have shown that the T_m calculated from CD thermal denaturation profiles is proportional to the peptide equilibrium binding constant (*23*). Our CD thermal denaturation experiments of class I MHC complexes with HER-2/neu-derived peptides displayed a range of T_ms. Interestingly, some of the complexes had T_ms that are at or below human basal body temperature (*24*). This result generated an interesting question. How can class I complexes fall apart at human physiological temperatures in vitro and stimulate an immune response in vivo? A hypothesis developed which states that physiological osmolytes stabilize class I MHC/peptide complexes against thermal denaturation in vitro and in vivo.

Many osmolytes are found in vivo as cosolvents (*25*). Salts, monosaccharides, and amino acids are typically found in micromolar to millimolar concentrations in the cellular environment. Most of these molecules have multiple functions inside the cell, but one function, which has been frequently neglected, is protein stabilization. Protein chemists have shown that specific osmolytes such as glycerol and sucrose can stabilize protein exposed to denaturing conditions at high concentrations (>1.0 M). Physiological concentrations of these agents do not typically have significant effects by themselves. We have performed numerous stability experiments with class I complexes using different cosolvents. The results were generally as expected; osmolytes stabilized class I MHC to thermal denaturation. The surprising result was that physiological concentrations of NaCl demonstrated the largest stabilization. We hypothesize that the stabilization is partly due to the increase of β_2m binding to heavy chain and partly due to stabilization by Debye−Hückel charge screening.

Table 1: Effect of 150 mM NaCl on the T_m of Class I MHC/Peptide Complexes[a]

MHC allotype	peptide	T_m (°C) (0.0 mM NaCl)	T_m (°C) (150 mM NaCl)	ΔT_m (°C)
A2	IISAVVGIL	36.2	60.0	23.8
A2	IISAVVGIV	38.8	59.8	21.0
A2	KISAVVGIL	41.6	55.9	14.3
A2	ILKEPVHGV	50.0	58.6	8.6
A2	YLKEPVHGV	56.0	61.7	5.7
A2	FLKEPVHGV	55.0	60.0	5.0
A2	KTWGQYWQV	52.7	57.7	5.0
A2	IMDQVPFSV	49.5	56.5	7.0
A2	ITDQVPFSV	45.1	53.9	8.8
A2	MLLSVPLLL	53.1	55.9	2.8
A2	ALGIVCPIC	45.3	63.4	18.1
D^b	KAVYNFATM	43.7	53.7[b]	10.0
D^b	FAPGVFPYM	41.0	65.0[b]	24.0

[a] Thermal melts were performed on multiple pMHC complexes as described in the text. [b] These values are for 100 mM NaCl.

EXPERIMENTAL PROCEDURES

Synthetic Peptides. All peptides were synthesized by the Peptide Synthesis Facility at the University of North Carolina, Chapel Hill. The sequences of the peptides are given in Table 1. All peptides were purified by reverse-phase HPLC to greater than 95% purity, and the sequences were confirmed by matrix-assisted laser desorption ionization−time-of-flight spectrometry.

Production of Class I MHC/Peptide Complexes. pMHC complexes were prepared as previously described (*26*). Briefly, the extracellular portions of class I MHC heavy chain were produced in *E. coli* as inclusion bodies. Purified inclusion bodies were rapidly diluted in the presence of β_2m, individual peptides, and a chaotropic buffer consisting of 100 mM Tris-HCl, pH 8.0, 400 mM L-arginine, pH 8.0, 6 mM glutathione (10:1 mixture of reduced/oxidized), and a cocktail of protease inhibitors. Folded pMHC complexes were concentrated by ultrafiltration (Amicon, Inc., Beverly, MA) with a YM10 membrane. The concentrated protein was purified to homogeneity by gel filtration on a BIOSEP SEC-S2000 column (Phenomenex, Torrance, CA) in a running buffer of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and exchanged into 10 mM KH_2PO_4/K_2HPO_4, pH 7.5, using Centricon filtration devices (Amicon, Inc.). Buffer was exchanged at least 3 times (8000-fold dilution) to ensure the removal of any residual buffers or salts. For those experiments requiring β_2m alone, the β_2m inclusion body material was folded in vitro as described above for pMHC except that a 3000 MW cutoff membrane was used for concentration before purification on the gel filtration column.

K^d covalently bound to β_2m (SC-K^d) and single-chain K^d complexes (sBDH) were produced in CHO cells and purified as described previously (*27, 28*). The purified protein was concentrated and exchanged into 10 mM KH_2PO_4/K_2HPO_4, pH 7.5, using Centricon filtration devices (Amicon, Inc.).

Circular Dichroism (CD) Spectroscopy and Thermal Denaturation Measurements. Purified complexes were diluted to 4−12 μM protein with 10 mM KH_2PO_4/K_2HPO_4 with or without additional cosolvents. The pH of the resulting solution was monitored carefully due to the known shift in T_m of class I complexes at low and high pH (*29*). The thermal denaturation profile (melting curve) of class I complexes was collected by monitoring the change in the circular dichroic signal as a function of temperature as described previously (*9, 30*). Thermal scans were performed on an AVIV 62DS spectrophotometer (Aviv Associates, Lakewood, NJ) equipped with a Peltier-effect temperature controller using 0.1 cm path length cuvettes. Thermal denaturation data were typically collected at 218 nm with 1 °C intervals from 4 to 95 °C. All measurements were made at least 3 times from different preparations and averaged. Thermal denaturation curves were scaled between 0 and 1 to provide plots of the fraction unfolded versus temperature for analysis. The T_m of a class I complex is the temperature at which 50% of the molecules are unfolded. CD spectra were collected between 350 and 200 nm at 1 nm increments and 10 s averaging times. Five or more spectra were averaged for the final spectrum of each sample.

Cell Surface Class I Measurements. Measurements of cell surface class I molecules loaded with endogenous peptides were performed as previously described (*31*). Briefly, T2 cells (ATCC no. CRL-1992), that are defective in antigen presentation (*32*), were loaded with the peptide of interest by incubating the cells in the presence of 50 μM each of peptide and β_2m. Peptide-pulsed T2 cells were cultured in serum-free AIM V media in a 5% CO_2 environment with and without additional salts. Flow cytometry was performed on a Becton Dickinson FACScan (Lincoln Park, NJ). Propidium iodide insensitive cells were used for all experiments to be sure only live cells were analyzed. The conformationally specific antibodies BB7.2 (HLA-A2 specific) (*33*), W6/32 (class I α3 chain/β2m specific) (*34*), and HbV (HLA-A2/HIV peptide specific; Dr. Ralph Kubo, personal communication) were used as the markers of folded class I structure.

RESULTS

During our examination of the thermal stability of peptides recognized by CTL from the tyrosine kinase HER-2/neu, we found some of the peptide/MHC (pMHC) complexes have denaturation midpoints that are at, or below, human basal body temperature (*24*). Knowing that these complexes bind to their cognate TCR and elicit an immune response in vivo, we sought to determine what factors might stabilize the class I MHC complexes in vitro to extrapolate to in vivo conditions. Physiological osmolytes are well-known as stabilizers (or destabilizers) of protein structure (*35*) as such were reasonable candidates as stabilizers of class I MHC against thermal denaturation. Therefore, the effect of cosolvents on the thermal stability of class I MHC molecules was examined.

Various osmolytes including polyalcohols such as glucose and PEG, amino acids, and amino acid derivatives such as glycine and betaine, and salts such as NaCl were each added to purified recombinant proteins, and the thermal stability was measured by circular dichroism. Since the yield of

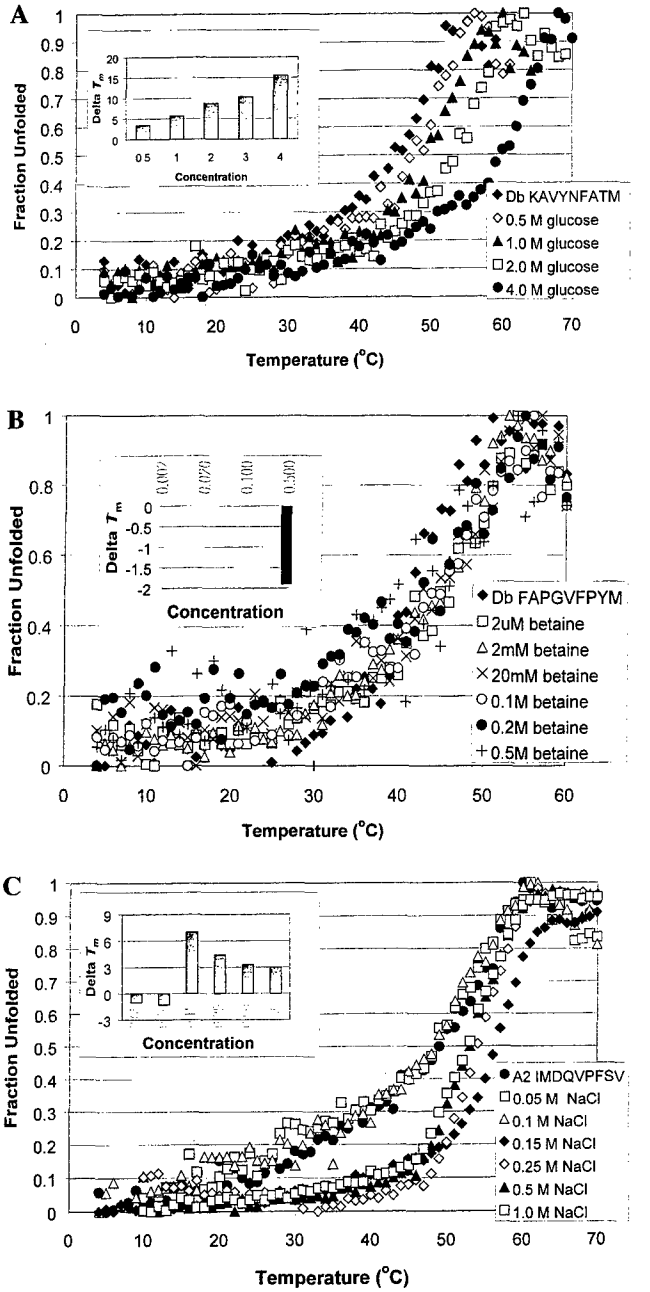

FIGURE 1: Class I MHC is stabilized against thermal denaturation by cosolvents. Thermal denaturation profiles of class I MHC in the presence of the indicated concentration of cosolvent. (A) Effect of added glucose. D^b KAVYNFATM folded in vitro was denatured in the presence of the indicated concentrations of glucose. (B) Effect of the amino acid analogue betaine. D^b FAPGVFPYM was thermally denatured in the absence of the indicated concentrations of betaine. (C) Effect of NaCl. A2 IMDQVPFSV was thermally denatured in the presence of the indicated concentrations of NaCl. The inset panels describe the difference in the calculated T_m from the T_m observed in the absence of added cosolvent. Each curve is the average of three independent experiments using 4−12 μM protein.

protein folded in vitro depends on the affinity of the peptide [(*36*) and unpublished observations], we used complexes that contained moderate- to high-affinity peptides for most analytical tests. Figure 1 shows representative results for glucose, betaine, and NaCl. Glucose shows a typical stabilization to thermal denaturation at increasing concentration (Figure 1A). Sucrose shows a similar stabilization at half the concentration. That observation is consistent with its

2-fold increase in volume relative to glucose (data not shown). Betaine showed very little effect at low concentration and a destabilizing effect at higher concentrations (Figure 1B). Glycine shows a similar destabilizing phenotype (data not shown). Interestingly, NaCl did not appear to give an easily interpretable result (Figure 1C).

Unlike the results seen with polyols and amino acids, titrations with NaCl from 5 μM to 1 M show that there is an optimal concentration of NaCl (150 mM for human class I MHC, 100 mM for murine class I MHC) for thermal stabilization. The titration shows an initial spike of stabilization at low concentration, followed by a relative destabilization, finishing with stabilization again at very high concentrations. The stabilization at high concentrations of NaCl (>0.5 M) is what has been seen many times previously for other proteins (*35*). The large increase in thermal stability at low salt concentration has not been observed previously. These effects were consistently seen for A2 and HLA-Aw68. The results seen for polyols, amino acids, and salts are not constrained to a specific MHC with a specific peptide; they are found with different peptides and different MHC molecules (data not shown). The degree of stabilization changes, but the concentration trends are the same for any class I MHC with one exception. The murine class I MHC molecule D^b showed maximum stabilization at 100 mM NaCl, not 150 mM as was seen for the human pMHC (data not shown).

We typically examine denaturation of these complexes at 218 nm. An uninteresting explanation for this effect would be if the shift in denaturation of all these pMHC complexes were due to artifactual wavelength-specific stabilization. Therefore, wavelength scans of class I MHC/peptide complexes with and without 150 mM NaCl were performed at different critical temperatures. There were no significant differences between the CD spectra of pMHC complexes at 4, 37, and 50 °C with or without 150 mM NaCl present (data not shown). There were small differences between the two conditions at 4 or 95 °C, and this observation was attributed to the solubility differences of these denatured complexes at the elevated temperatures. Denatured class I MHC complexes in the absence of NaCl are slightly soluble, while those with NaCl are not soluble as evidenced by a white precipitate at the bottom of the cuvette. These data suggest that there are no gross rearrangements of the secondary structure as a function of the NaCl concentration, and the effect is due to protein denaturation at different temperatures (data not shown). Additionally, the thermal denaturation of pMHC is reversible by the following criteria. Protein that has been heated to 90 °C for 5 min and cooled retains the same spectra as the native protein. Furthermore, the resulting protein gives the same T_m if denatured again. The denaturation is locally reversible as the T_m does not shift if a scan rate twice as long is used. Three-fold differences in pMHC concentration had no effect on T_m. However, it is not fully reversible. Much of the protein is not recovered from the first denaturation curve, and the solution must be filtered to remove the insoluble material before the second experiment is performed.

We also sought to confirm that this thermal stabilization is not artifactual by using an entirely different assay. We examined the condition of class I MHC on the surface of cells in the presence of different concentrations of NaCl by

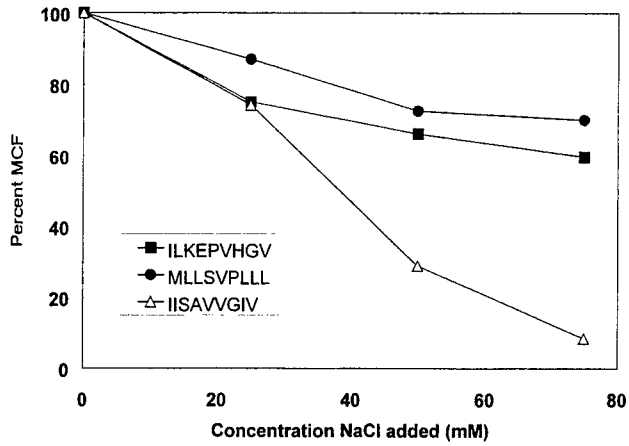

FIGURE 2: Destabilizing effect of NaCl above 150 mM is also seen on the surface of cells. Cell surface assembly assays of A2 were performed on T2 cells with the indicated peptides in the presence of 25, 50, and 75 mM NaCl added to the cell culture medium. As additional NaCl was not perfectly tolerated by the cells, live cells were analyzed by gating on propidium iodide negative cells.

flow cytometry. T2 cells were chosen because they do not have functional peptide transporters, and thus a large fraction of the class I complexes bind exogenous peptide on the cell surface (*32*). NaCl could not be removed from the medium because the cells would die, but it could be added to the media in small amounts. Based on the data described above (Figure 1C, inset panel), NaCl concentrations above 150 mM reduce the T_ms relative to 150 mM NaCl. The hypothesis was that additional NaCl would lower the T_ms of class I on the surface of cells, resulting in fewer available complexes. First, we tested how much salt the cells could tolerate. Additional NaCl was added in 25 mM increments to AIM V serum-free medium and T2 cell viability assessed. The cells tolerated up to an additional 75 mM NaCl added to the medium over the course of the experiment (~24 h). Next, cell surface complexes of A2 were measured as a function of added NaCl. As can be seen from Figure 2, the additional NaCl reduced the number of A2 complexes on the surface of T2 cells. Different peptides bound in the pMHC showed different degrees of destabilization on the T2 cells depending on the concentration of added NaCl (Figure 2). The most affected were cells incubated with IISAVVGIV, and the least affected were cells incubated with MLLSVPLLL. Therefore, the magnitude of the effect on the surface of T2 cells correlated directly with the magnitude of the effect on T_m as measured by CD (Table 1). The effect was also not due to the hydrophobicity of the peptide, because these two peptides are very similar hydrophobicities. The shifts in fluorescence were also not due to a reduction in affinity of the BB7.2 antibody for HLA-A2, because similar effects were seen with two different antibodies that bind in distinctly different locations on the class I molecule (antibodies W6/32 and HbV, data not shown).

Having concluded that the NaCl effect on pMHC is not an artifact of the CD experiment, we reexamined the effect of NaCl on a well-studied protein, hen egg white lysozyme (HEL), and also β_2m. Figure 3 shows the change in T_m for HEL and β_2m in the presence of increasing concentrations of NaCl. There are relatively small, nearly monotonic positive changes in T_m up to 500 mM NaCl for HEL and larger, positive changes at 1.0 and 2.0 M NaCl. There was no peak of stabilization, and the observed stabilization of HEL at 150

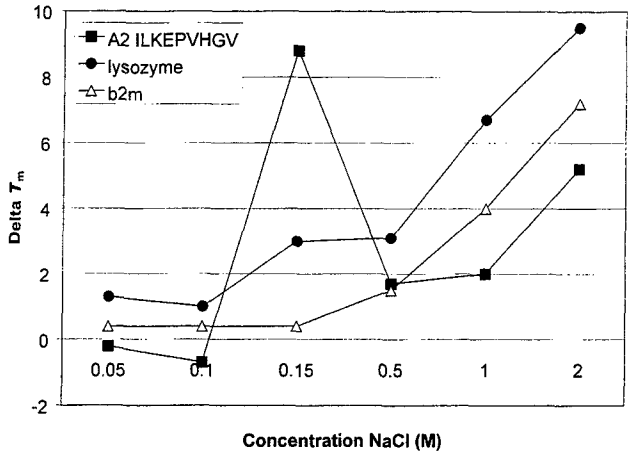

FIGURE 3: Effect of NaCl on class I MHC is not generalizable to other proteins. The indicated amounts of NaCl were added to samples of A2 ILKEPVHGV, lysozyme, or β_2m and the thermal denaturation profiles determined. Delta T_m is the calculated T_m in the absence of NaCl subtracted from calculated T_m at the indicated concentration.

mM NaCl (~2 °C) was not nearly as large as most of the effects on class I MHC (Table 1). There was no observed change in the T_m of β_2m until 0.5 M NaCl was added.

The effects of other salts on class I MHC were also examined. Figure 4A demonstrates that KCl shifts the T_m of complexes to greater temperatures than NaCl. However, the maximum was observed to be 150 mM, suggesting that ionic strength may be part of the reason the protein is stabilized. Figure 4B shows the effects of Na_2SO_4 on class I complexes. At an equivalent point of ionic strength, the effects of NaCl, KCl, and Na_2SO_4 are very similar, but different in intensity. Additionally, some salts shifted the T_ms of complexes to lower values. For example, at 150 mM NaSCN, the A2 ILKEPVHGV complex was so disrupted an accurate T_m could not be assessed. Also, LiBr at 150 mM shifted the T_m of the A2 ILKEPVHGV complex by approximately −20 °C (data not shown). In a survey of other biologically important ions, we tested the effect of 5 mM Ca^{2+} ($CaCl_2$, estimated to be the physiological concentration of Ca^{2+} in the ER) on the thermal denaturation of pMHC [in MES buffer due to the precipitation of $Ca_2(PO_4)_3$]. There was no effect on the T_m of pMHC with 5 mM Ca^{2+} (data not shown). NaCl does not change the size, shape, or oligomeric state of pMHC as determined by dynamic light scattering and sedimentation velocity experiments (data not shown).

Determining how NaCl could exert its effects on class I MHC is made difficult because of the nature of the complex. Most experiments looking at cosolvent effects were performed on small single-domain proteins. If we consider the pMHC ternary complex as a single unit, the effect would be due to changes in protein solvation, discrete ion binding to the protein, or charge screening on the protein. However, we may consider class I MHC to be a heavy chain with two ligands (peptide and β_2m). If we examine the molecule that way, the stabilization effect could be due to stabilization of either peptide binding or β_2m binding to the heavy chain as each ligand has been shown to be required for complex formation [(*37, 38*) and unpublished data].

Does the nature of the peptide affect the degree of thermal stabilization by NaCl? A summary of different peptides and different complexes tested for thermal stability by CD is

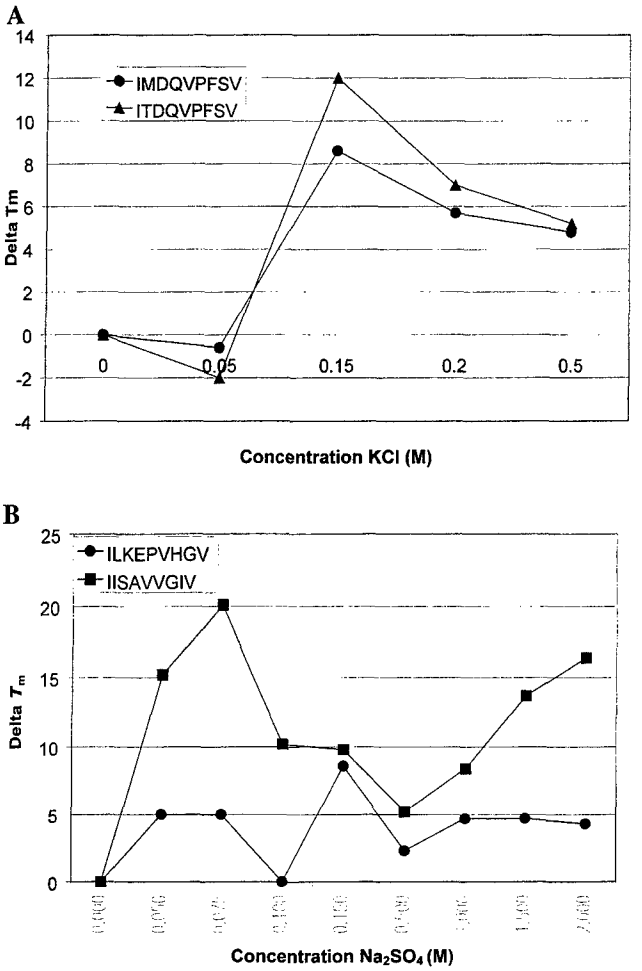

FIGURE 4: Effect generally correlates with the Hofmeister series for anions. Change in melting temperature of the class I complexes versus concentration of KCl and Na_2SO_4. (A) KCl stabilization of class I complexes of A2 bound to IMDQVPFSV or ITDQVPFSF. (B) Effect on thermal stabilization of pMHC (A2 and ILKEPVHGV or IISAVVGIV) in the presence of the indicated amounts of Na_2SO_4.

shown in Table 1. All of the peptides conform to the known peptide-binding motif for its allotype. Therefore, differences are not due to specific interactions with the peptide-binding cleft. However, peptide hydrophobicity could play a role. One can imagine that the addition of salt to the solvent would drive the association of peptide with the heavy chain. If peptide release is an important early step in thermal denaturation, then the stabilization would be greatest for nonpolar peptides and least for more polar peptides. Figure 5A shows a plot of the calculated hydrophobicity of a series of peptides versus the observed increase in T_m. There is clearly not a simple relationship suggesting that the hydrophobicity of the peptide does not affect the stability of the complex and that peptide release is not affected by the polarity of the solvent. This has been seen before for HLA-Aw68 (*39*). Additionally, if peptide dissociation were to affect the thermal stability of the protein, the pMHC in the cuvette should show a higher T_m in the presence of excess additional peptide. Addition of 5 μM IISAVVGIL excess peptide to A2 IISAVVGIL does not change the T_m (data not shown). Therefore, thermal denaturation is not keyed to peptide dissociation. Figure 5B shows a plot of the initial T_m versus the degree of stabilization. It is clear from the 77% correlation coefficient that the degree of stabilization

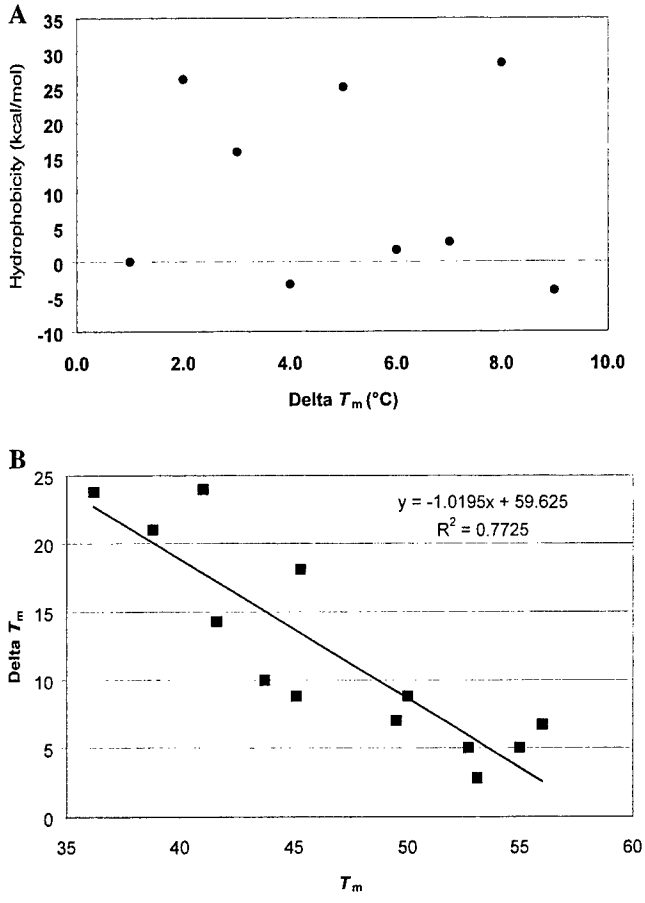

FIGURE 5: NaCl-induced change in T_m depends on the initial T_m and not on the hydrophobicity of the peptide ligand. (A) The plot of peptide hydrophobicity versus the delta T_m demonstrates that the change in T_m of class I MHC complexes is not dependent on the peptide sequence. Hydrophobicities are calculated from the sum of the free energies of transfer of the individual amino acids from cyclohexane to water (*50*). (B) Plot of the change in T_m versus initial T_m shows that the change in T_m is dependent on the initial T_m. The peptides and their respective T_ms are listed in Table 1.

is maximal for the poorest binding peptides. These data suggest that the stabilization by NaCl is not primarily due to any effect of the composition of the peptide, but it is related to how well the complex is held together.

Another ligand-binding scenario is that NaCl stabilizes the interaction of β_2m with the complex and thereby increases the thermal stability. The dissociation of β_2m has been described as a good measure of the dissociation of the peptide (*40*). Thus, it appears that peptide and β_2m dissociate from the heavy chain in a dependent fashion. The affinity of β_2m does not have a great effect on the T_m. Human β_2m has a 3-fold greater affinity for murine heavy chain than does murine β_2m (*41*). A murine class I MHC complex D^b folded with human β_2m and a low-affinity peptide FAPGVFPYM has a slightly higher T_m than the complex folded with murine β_2m (47 versus 42 °C) (Figure 6A). If β_2m dissociation were the first step in pMHC complex denaturation, addition of excess β_2m should shift the equilibrium to higher T_ms. However, if an excess of β_2m is added to the cuvette that contains the pMHC to be denatured, it does not increase the T_m (Figure 6B). Addition of NaCl to a mixture of pMHC and a 2-fold molar excess of β_2m and 100 mM NaCl stabilizes the protein to the degree seen in the absence of excess β_2m. To examine how a very large increase in affinity

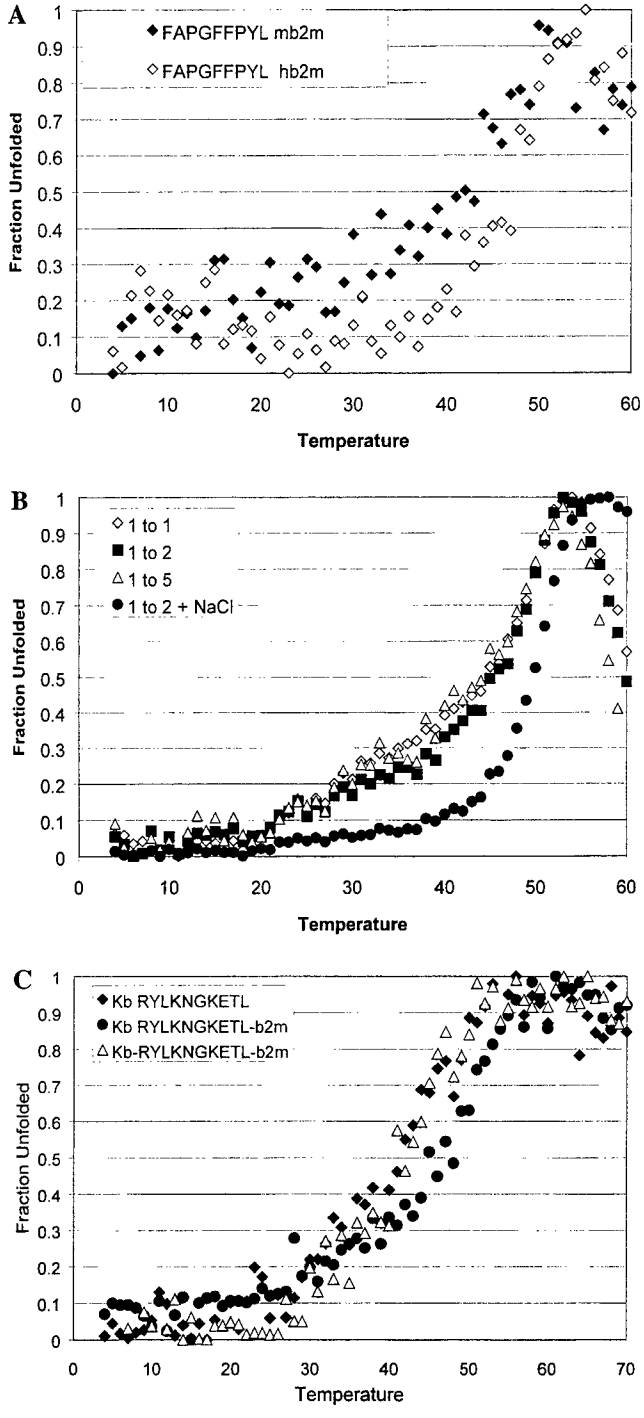

FIGURE 6: Increased association of β_2m increases the thermal stability, but not to the levels seen by the addition of NaCl. (A) D^b FAPGFFPYL has a higher T_m with hβ_2m bound than with mβ_2m. (B) The addition of molar excess of β_2m does not increase the T_m of pMHC. D^b KAVYNFATM was thermally denatured in the in the absence or presence of 1:1, 2:1, or 5:1 molar excess of mβ_2m added or in the presence of 2-fold molar excess β_2m plus 100mM NaCl. (C) Covalent addition of β_2m to the protein (Kb RYLKNGKETL-β_2m) increases the thermal stability of pMHC. However, covalent addition of peptide to make a fully covalent complex (Kb-RYLKNGKETL-β_2m) is not as thermally stable as the β_2m-attached and peptide-"free" complex.

would affect the thermal stability, we looked at protein that has β_2m covalently attached to the heavy chain (*42*). As can be seen in Figure 6C, the protein has a higher T_m compared to the noncovalently associated material (shift of ~5 °C). As the covalently linked protein has been produced in

mammalian cells and is glycosylated, we cannot exclude that the protein may be stabilized by the glycosylation. However, a complex that has covalently linked peptide and covalently linked β_2m (*43*) has a nearly identical T_m to that of the noncovalently associated ternary complex folded from material made in *E. coli* (Figure 6C). The fully covalent complex is most likely destabilized with respect to the covalent β_2m because the antigenic peptide extends from the peptide-binding groove. This has already been shown to be destabilizing due to the disruption of a conserved hydrogen bond with the peptide carboxyl terminus (*3*).

DISCUSSION

Class I MHC proteins have a simple role: to bind peptides in the endoplasmic reticulum and transport them to the cell surface. Differentiation of immunogenic versus ignored peptides is performed by TCR on the surface of circulating $CD8^+$ T cells. Most previously identified immunogenic viral epitopes bind to class I MHC well. However, peptides that bind poorly to class I MHC in vitro are also recognized by $CD8^+$ T cells in vivo (*24*). To resolve the paradox of the in vivo biological activity (i.e., T cell activation) with these in vitro physical measurements, we looked for other molecules that could stabilize proteins in vivo. This led to the examination of osmolytes as stabilizing agents and to the surprise observation that class I MHC stabilization was keyed to 150 mM NaCl.

When we began these studies, we expected to see modest increases in stability at high concentrations that we would extrapolate back to physiological conditions. As was anticipated, class I MHC molecules are stabilized by polyols such as glucose (Figure 1A) and sucrose (data not shown) at high concentrations. Other polyols, such as glycerol and poly(ethylene glycol), are destabilizing (data not shown). Amino acids (glycine data are not shown) and amino acid derivatives (Figure 1B) are destabilizing. NaCl (Figure 1C) and KCl (Figure 4A) are stabilizing at molar amounts compared to the absence of NaCl as is seen for other proteins. However, in a manner not seen before, class I MHC is maximally stabilized to thermal denaturation at physiological concentrations of added NaCl.

The mechanism of class I MHC stabilization to thermal denaturation by NaCl is not clear. There are two different levels of potential stabilization to consider. The first treats the trimolecular complex as the sum of its parts. In that scheme, peptide and β_2m may be considered as ligands binding to the heavy chain. Enzyme stability has been shown to be increased by increases in ionic strength. This phenomenon has been attributed to an increase in the affinity of the ligand for the protein due to the hydrophobic effect. The observation that the increased thermal stability in the more polar environment does not correlate with peptide hydrophobicity suggests that the release of peptide is not the critical event in thermal denaturation of this complex. Similarly, changes in β_2m affinity can increase thermal stability, but the effect is very small and does not account for the large change in thermal stability in the presence of salt. Last, in both of these instances, if the effect were due to a change in partitioning of the parts of the protein due to polarity of the environment, we would expect the effect to saturate, but not peak and decline as we observe here.

The second potential mechanism of stabilization treats the trimolecular complex as a single unit. This is not necessarily a poor assumption because the thermal denaturation is two-state. If the protein is a single unit, there are three potential explanations for this stabilization by salts: changes in protein solvation, discrete ion binding, and Debye–Hückel charge screening. 150 mM NaCl is considered to be a dilute aqueous salt solution and is not considered to contribute significantly to the protein solvation (hydrophobic) effect (*44, 45*). However, if protein solvation is a factor here, anions should dominate, and the effect should follow the Hofmeister series (*46*). If discrete ion binding plays a role, the effect should follow the electroselectivity series of anions binding to ion-exchange resins (*47*). In the absence of clear evidence of either of the other two possibilities, we would propose that Debye–Hückel screening is important, but it is normally seen in solutions below 0.1 M (*48*).

All of these factors probably contribute to the observed phenotype of class I MHC. While it is difficult to dissect the contributions from each of these factors from these experiments, we can make the following statements. The effect of the various ions does not appear to follow the electroselectivity series. It does follow the Hofmeister series, but as stated above, this effect is not typically described for dilute solutions. The most reasonable explanation is Debye–Hückel screening because stabilization (a) roughly follows ionic strength (compare NaCl versus Na_2SO_4 effects), (b) is observed at low concentrations of salts, and (c) is primarily associated with the cation (KCl stabilizes more than an equimolar amount of NaCl). Therefore, at concentrations up to 150 mM NaCl, a relatively long-range destabilizing charge within the protein is shielded (Debye length ~8 Å). At concentrations above 150 mM (shorter Debye length), the situation is reversed, and a stabilizing charge is shielded. This would make the protein less stable.

Thermal denaturation studies have been shown to be an accurate way to evaluate peptide binding to class I MHC molecules (*9, 30*), and the derived T_ms have been shown to be proportional to the peptide equilibrium dissociation constant (*23*). We have performed many experiments comparing T_ms and relative binding constants on the surface of T2 cells. The relative binding constant that we derive has shown an excellent correlation with T_m as long as there are no cosolvents present in the CD buffer (unpublished data). However, this correlation must be qualified. As demonstrated here, it clearly does not hold true under all conditions. In the absence of salts, measurement of the T_m of the class I ternary complex by CD spectroscopy is indicative of the inherent affinity of the peptide for class I receptors (*23*). In the presence of salts, the T_m no longer reflects the peptide affinity for the class I receptor but represents the overall thermal stability of the class I ternary complex. Therefore, it seems likely that the different experiments are measuring two different processes. In the absence of salt, the denaturation of the complex is tied to the affinity of the peptide. In the presence of salts, the T_m is not related to peptide binding affinity. As can be seen in Table 1, the T_m is basically the same (60 °C) for each complex independent of the peptide bound (the error in T_m is roughly 1 °C). Thus, the process measured by CD in the presence of NaCl is different from the process measured during equilibrium peptide binding measures. The CD experiment in the presence of NaCl

apparently evaluates the inherent protein stability independent of the peptide bound.

Class I molecules have a difficult role in the adaptive immune response. They must bind a diverse set of peptides with different affinities and low specificity. Anchor residues reduce the number of peptides that may bind and limit the repertoire of presentable peptides. An examination of the structure of class I MHC shows that the peptidic termini are buried deep in pockets in the peptide-binding groove. It appears likely that a large conformational change would be required to release peptide. Some evidence of this phenomenon has been seen with specific antibodies (*49*). Here we show that class I MHC is less soluble in the presence of 150 mM NaCl than it is in the absence of NaCl. This may be an important aspect of the role of class I MHC in the immune system. Class I MHC must hold potentially antigenic peptides for long half-lives in order to allow the clonotypic T cells time to engage the ligands. $CD8^+$ T cells that recognize these complexes lyse the cells presenting the peptide ligand on class I MHC. Thus, it is critical that the viral or cancer indicating peptide be only presented on the infected or cancerous cells; therefore, peptide exchange from one class I molecule to another at the cell surface is undesirable. Here we show that class I molecules denature in the absence of peptide, which makes them incapable of binding another peptide. Additionally, this is another mechanism to fully exploit the available repertoire of peptides; the class I MHC molecules take advantage of the cellular environment. In particular, we have shown salts can stabilize the class I MHC ternary complex against thermal denaturation. Therefore, researchers measuring peptide-binding affinity by CD for development of immunotherapeutics should be careful to observe that the link between affinity and T_m does not hold true in the presence of NaCl. However, these insights allow the selection of peptides once classified as unusable because of their low T_m. These results resolve the dilemma of how class I MHC molecules loaded with extremely low-affinity peptides can have subphysiological T_ms yet are able to stimulate T cells in the immune system.

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T Cell Activity After Dendritic Cell Vaccination Is Dependent on Both the Type of Antigen and the Mode of Delivery[1]

Jonathan S. Serody,[2,3*†] Edward J. Collins,[2†‡] Roland M. Tisch,[†] Jennifer J. Kuhns,[†] and Jeffrey A. Frelinger [†]

Previous work in both human and animal models has shown that CTL responses can be generated against proteins derived from tumors using either peptide-pulsed dendritic cells (DCs) or nucleic acids from the tumor transfected into autologous DCs. Despite the efficacy of this approach for vaccine therapy, many questions remain regarding whether the route of administration, the frequency of administration, or the type of Ag is critical to generating T cell responses to these Ags. We have investigated methods to enhance CTL responses to a peptide derived from the human proto-oncogene HER-2/neu using mice containing a chimeric HLA A2 and $H2K^b$ allele. Changes in amino acids in the anchor positions of the peptide enhanced the binding of the peptide to HLA-A2 in vitro, but did not enhance the immunogenicity of the peptide in vivo. In contrast, when autologous DCs presented peptides, significant CTL activity was induced with the altered, but not the wild-type, peptide. We found that the route of administration affected the anatomic site and the time to onset of CTL activity, but did not impact on the magnitude of the response. To our surprise, we observed that weekly administration of peptide-pulsed DCs led to diminishing CTL activity after 6 wk of treatment. This was not found in animals injected with DCs every 3 wk for six treatments or in animals initially given DCs weekly and then injected weekly with peptide-pulsed C1R-A2 transfectants. *The Journal of Immunology,* **2000, 164: 4961–4967.**

A number of different investigators have shown both in vitro and in vivo that $CD8^+$ CTL can be generated against tumor proteins (1–12). There has been increased interest recently in the use of autologous dendritic cells (DCs)[4] loaded with peptides, tumor lysates, or transfected with DNA or RNA to generate T cell responses in patients with various malignancies (3, 5–8, 10, 13–16). Despite modest success in treating patients with metastatic melanoma and prostate cancer, many questions remain unanswered regarding the mode and timing of delivery of tumor Ag-loaded autologous DCs cells and the type of Ag to be used in these treatments.

One of the difficulties in this area is the lack of animal models to evaluate DC function. Class I MHC proteins bind peptides that are typically 8–10 aa in length and generated by degradation of cytosolic proteins by the proteosome (17–22). MHC molecules from different animal species bind different sets of peptides. Thus, studies to optimize DC activity in mice using mouse MHC proteins may have little applicability to enhancing CTL responses to epitopes derived from human tumors.

HER-2/neu is a proto-oncogene that is overexpressed in ~33% of patients with breast cancer (23, 24). Previous investigators have shown that aa 654–662 from HER-2/Neu (termed GP2 in this manuscript) can bind to HLA-A2 and initiate a T cell response against adenocarcinoma cells of the breast, ovary, and pancreas (25, 26). To overcome the problems associated with both human epitopes not binding to mouse MHC molecules and mouse $CD8^+$ T cells not interacting well with the $\alpha 3$ domain of human MHC proteins, we generated transgenic mice expressing the $\alpha 1$ and $\alpha 2$ domains of HLA-A2 and the $\alpha 3$ domain of $H2K^b$. Using these mice, we have explored the influence of the type of Ag and the route and timing of delivery of autologous DCs in the generation of CTL responses against the GP2 epitope from HER-2/Neu.

Materials and Methods

Cell lines

T2 (174 × CEM.T2), HmyC1R-neo (C1R-neo), and HmyC1R-B*0702 (C1R-B7) cell lines were provided by Dr. Peter Cresswell (Yale Medical Center, New Haven, CT) (27). HmyC1R-A*0201 (C1R-A2) cells were described previously (28). All cells were grown in RPMI 1640 supplemented with 10% (v/v) FBS, 5 mM L-glutamine, and 5×10^{-5} M 2-ME (R10). DCs were generated from mouse bone marrow by flushing the femur and tibia of individual mice with R10 and culturing the isolated cells in low adherence plates (Costar, Cambridge, MA) in the presence of GM-CSF (10 ng/ml), and IL-4 (1000 U/ml; Endogen, Woburn, MA). GM-CSF and IL-4 were added to the medium at the same concentration on days 3 and 7. Additionally on day 7, TNF-α (10 ng/ml; Endogen) was added to the medium, and the nonadherent cells were collected on day 12 and analyzed by flow cytometry before use in vaccination protocols. Human EBV-lymphoblastoid cell lines (EBV-LCL) were generated as previously described (29). HLA $A2^+$ and $B7^+$ EBV-LCL were used in these experiments.

Flow cytometry

On day 12, expanded DCs were characterized for the expression of specific surface markers by flow cytometry as described previously (30). Cells were stained with the following Abs: FITC-CD3ϵ (clone 145-2C11 IgG1), FITC-CD4 (clone GK1.5 IgG2b), FITC-CD8α (clone 53-6.7 IgG2a), PE-CD11b (clone M1.70 IgG2b), FITC-CD11c (clone HL3 IgG1), FITC-CD14 (clone rmC5-3 IgG1), PE-CD80 (clone 16-10A1 IgG2), PE-CD86 (clone GL1 IgG2a), FITC-I-A^b (clone AF6-120.1 IgG2a), and FITC-Mac-1

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[4] Abbreviations used in this paper: DC, dendritic cells; BFA, brefeldin A; i.d., intradermally; T_m, temperature at which 50% of protein is denatured; LCL, lymphoblastoid cell lines; APL, altered peptide ligand.

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(all Abs from PharMingen, San Diego, CA). DCs were analyzed using a FACScan (Becton Dickinson, Franklin Lakes, NJ) with Cicero software (Cytomation, Fort Collins, CO). Mature DCs were characterized by a 100-fold increased expression of CD80 or CD86 and class II compared with adherent macrophages, and in addition >90% of the cells had to express both CD11b and CD11c. For the vaccination protocols, 75–85% of the cells injected were DCs by the previous characterization.

Preparation of HLA-A2/peptide complexes

Residues 1–275 of HLA-A2 and human β_2m were expressed in *Escherichia coli* as inclusion bodies, purified, and folded as described previously (31).

Synthetic peptides

All peptides were synthesized by the Peptide Synthesis Facility at the University of North Carolina (Chapel Hill, NC). The sequence of the wild-type peptide (GP2) is IISAVVGIL. Substitutions made at peptide positions 2 and 9 are given in single-letter code abbreviations. The peptides were purified to >95% purity by reverse phase HPLC, and identity was confirmed by mass spectrometry. All peptides were dissolved in 100% DMSO at 20 mg/ml by weight.

Determination of thermal stability

HLA-A2/peptide complexes were exchanged into a 10 mM KH_2/K_2HPO_4 buffer, pH 7.5, and adjusted to a final protein concentration of 4–12 μM. The change in circular dichroic signal at 218 nm was measured as a function of temperature from 4–95°C on a Peltier temperature-controlled AVIV 62-DS spectropolarimeter (Aviv Associates, Lakewood, NJ). The final melting curve was the average of at least three experiments for each HLA-A2/peptide complex. T_m values were calculated as the temperature at which 50% of the complexes were denatured using a two-state denaturation model (32).

Cell surface half-life assay

The determination of cell surface half-lives ($t_{1/2}$) of A2/peptide complexes was performed as described previously (33). Briefly, 2.5×10^6 T2 cells were incubated overnight in AIM V serum-free medium at 37°C in 5% CO_2 in the presence of 50 μM peptide. To block the egress of new A2 molecules to the surface, cells were incubated at 37°C in 5% CO_2 in RPMI 1640, 15% FCS, and 10 μg/ml brefeldin A (BFA; Sigma, St. Louis, MO). This concentration of BFA is toxic to the cells; therefore, after 1 h the cells were transferred to RPMI 1640, 15% FCS, and 0.5 μg/ml BFA. At the indicated time points, 2.5×10^5 cells were removed, incubated with BB7.2, and analyzed by flow cytometry as described above for cell surface stabilization assay. Each time point is evaluated as the mean fluorescence with peptide minus the mean fluorescence without peptide and normalized to the maximal level of fluorescence (at time zero) for each peptide.

Construction of A2K^b transgenic mice

An HLA-A**0201*/H2K^b (A2K^b) fusion gene was constructed from a genomic clone of HLA-A**0201* and H2K^b. A *Hin*dIII-*Bgl*II fragment encoding the promoter sequence, leader sequence, and exons 1–3 of HLA-*A*0201* was ligated to a *Bam*HI fragment encoding exons 4–7 and 5′-flanking sequences of H2K^b. The fusion construct was sequenced for verification and microinjected into embryos derived from FVB/n mice. Founder mice were assessed by surface expression of HLA-A2 using flow cytometry on peripheral blood cells. All animal work was performed under protocols approved by the Division of Laboratory Animal Medicine at the University of North Carolina.

*CTL activity in A*0201 K^b transgenic mice (A2K^b)*

DCs were cultured from bone marrow cells isolated from A2K^b transgenic mice in R10. After the addition of recombinant cytokines as described previously, DCs were cultured in six-well nonadherent plates. After 12 days, an aliquot of the expanding cells was analyzed by flow cytometry as previously described. Another aliquot of cells (1×10^6) was incubated with 10 μg of GP2, L9V, or I2L peptide in PBS and 0.5% mouse serum. DCs (5×10^5) were injected either intradermally (i.d.; footpad) or s.c. (base of the tail) or into the lateral tail vein (i.v.) of A2K^b mice in the same manner at specific intervals (weekly or every 3 wk). After the indicated number of vaccinations, mice were sacrificed. For local s.c. or i.d. injections the draining lymph nodes were removed, and lymphocytes were isolated and tested for lysis of peptide-pulsed target cells by a conventional ^{51}Cr release assay. For i.v. inoculation, splenocytes were removed and tested for CTL activity.

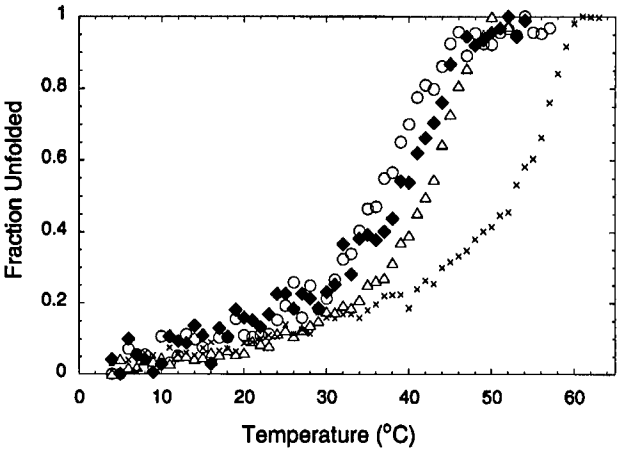

FIGURE 1. The HER-2/Neu-derived peptide IISAVVGIL (GP2) binds poorly to A2, as shown by circular dichroism (CD). The CD signal of A2 complexed with GP2, variants at anchor positions, or the peptide MLLSVPLLL was measured at 218 nm as a function of temperature from 4–70°C. Even though the GP2 and MLLSVPLLL peptides have similar hydrophobicities, the GP2/A2 (○) complex is considerably less stable than the MLLSVPLLL/A2 (×) complex, as seen by a lower melting temperature (T_m), 36 vs 54°C. Modification of anchor residues (P2 and P9) caused an increase in the melting temperature. Higher T_m values for peptides with substitutions at P2 (I2L; △) and P9 (L9V, ◆) of GP2 (○) indicate that substitutions at these positions increase the complex's stability.

The percent specific lysis for CTL assays was determined using/ the following formula: % specific lysis = $[(cpm_{sample} - cpm_{spontaneous})/(cpm_{total} - cpm_{spontaneous})] \times 100$. Lytic units were calculated using a program provided by G. Ferrari (Duke University, Durham, NC). The LU_{20} value is the number of lytic units per million cells required to yield 20% specific lysis.

Long-term responses

A2K^b mice were injected s.c. with autologous DCs every week for 3 wk. Subsequently, half the mice were injected weekly s.c. with peptide-pulsed DCs for a total of seven vaccinations. A second cohort of animals was injected weekly s.c. with peptide-pulsed C1R-A2 cells. Following the sixth and seventh injections, mice were sacrificed, and CTL assays were performed on isolated splenocytes. A third cohort of A2K^b transgenic mice was treated every 3 wk s.c. with peptide-pulsed DCs for a total of six

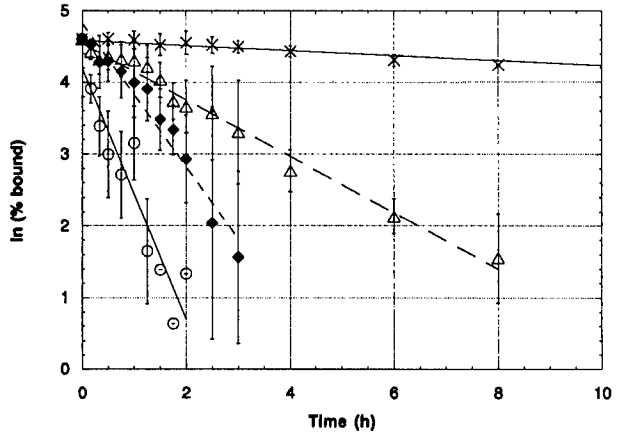

FIGURE 2. GP2 poorly stabilizes HLA-A2 on the surface of T2 cells. The cell surface half-lives of A2 with GP2 variants are increased with respect to that of wild-type GP2. T2 cells were incubated with 50 μM peptide overnight, washed, and treated with BFA to stop vesicular transport of A2 complexes. Aliquots of cells were removed at the indicated times, and cell surface A2 was measured by flow cytometry using the A2-specific mAb BB7.2. ○, GP2; △, I2L; ◆, L9V; ×, ML.

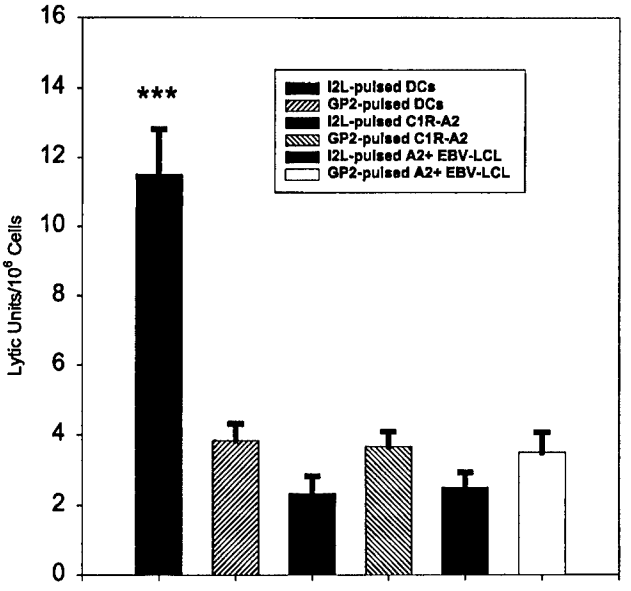

FIGURE 3. CTL activity from splenocytes after i.v. vaccination with wild-type or I2L-pulsed EBV-LCLs, C1R-A2 transfectants, or DCs. $A2K^b$ mice were vaccinated every 7–10 days i.v. four times with wild-type peptide or I2L pulsed onto DCs, EBV-LCL ($A2^+$), or C1R-A2 transfectants. After the fourth vaccination, mice were sacrificed, the spleen was removed, and red cells were removed using ACK lysis buffer. The remaining splenocytes were tested for lysis of C1R-neo, C1R-A2, or C1R-B7 target cells incubated with the wild-type peptide IISAVVGIL in a conventional ^{51}Cr release assay. No lytic activity was seen using C1R-B7 or C1R-neo cells as targets (data not shown). The data are expressed as lytic units per 10^6 cells and are pooled from two separate experiments, using four $A2K^b$ mice in each experimental group. Spontaneous release for all target cells was <10% of total release. ***, $p = 0.02$, difference between lytic activity using I2L-pulsed DCs compared with all other groups, by Mann-Whitney rank-sum test.

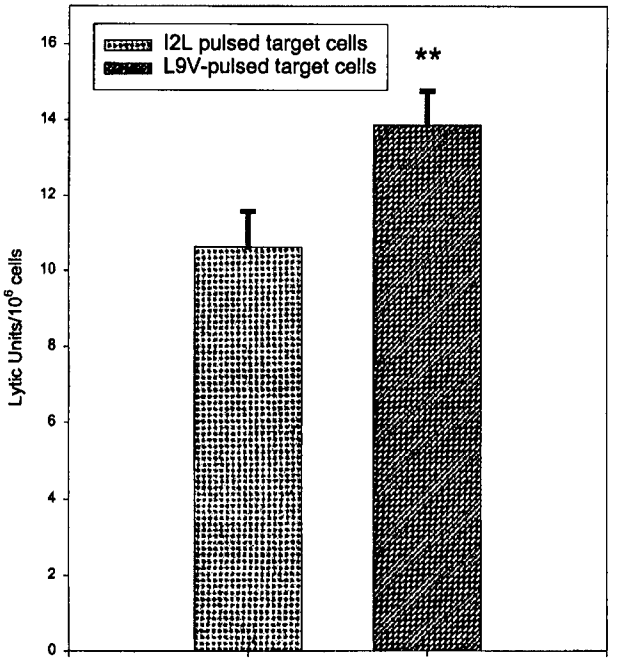

FIGURE 4. CTL activity is not affected by decreased affinity of L9V for HLA-A2. $A2K^b$ mice were vaccinated every 7–8 days with either 5×10^5 I2L-pulsed DCs or L9V-pulsed DCs i.v. After the fourth vaccination, the mice were sacrificed, and CTL assays were performed from isolated splenocytes as described above. The data are pooled from two separate experiments in which four or five mice were included in each group. Spontaneous release from all target cells was <10%. **, $p = 0.04$, by Mann-Whitney rank-sum test.

treatments. CTL responses were analyzed after s.c. vaccination from the draining lymph nodes.

Results

Identification of the APL by thermal stability

The T_m, which is the temperature at which 50% of protein is denatured, has been shown previously to be proportional to the free energy of peptide binding to class I MHC molecules (32, 34). Initially we examined the binding affinity of GP2 to the class I heavy chain HLA-A2. As shown in Fig. 1, the T_m for the GP2 peptide was 36.4°C. In comparison, the T_m for a high affinity peptide such as the A2 binding peptide from the signal sequence of calreticulin is ~54°C (Fig. 1). Because GP2 lacked the preferred amino acids found in the anchor positions of HLA-A2, we prepared peptides substituted with the preferred amino acids. Substitutions of amino acids in the anchor positions of the peptide stabilized the complex, as shown by an increase in the T_m of 2 and 6°C for the substituted peptides L9V and I2L, respectively. An increase of 6°C is significant and suggests a large increase in the half-life of the complex.

Stability of HLA-A2/GP2 complexes on T2 cells

We next measured the half-life of HLA-A2 complexes incubated with GP2, the APLs, and a strong binding peptide from calreticulin (ML) on T2 cells. This cell line has a large deletion of genetic material on chromosome 6, which encompasses the TAP1 and TAP2 genes (27). Thus, the T2 cell line does not efficiently present endogenous nonsignal sequence peptides, and the MHC complexes that egress to the surface readily disassociate in the absence of peptide. However, these complexes can be stabilized by addition of exogenous peptide. We incubated T2 cells in serum-free medium overnight with 50 μM of the individual peptides and measured the half-life of the HLA-A2 complex by flow cytometry. The calculated half-lives were 24 min, 42 min, 1.8 h, and 19.5 h, respectively, for GP2, L9V, I2L, and ML (Fig. 2). Thus, we confirmed that substitutions in the anchor positions of the GP2 peptide greatly enhanced the stability of MHC complexes in vitro.

CTL responses in $A2K^b$ mice

To evaluate whether the enhanced immunogenicity found in vitro using the APLs affected in vivo activity, peptide-pulsed DCs were administered to $A2K^b$ mice i.v., and CTL activity was determined from isolated splenocytes. Initially, the nature of the CTL response was evaluated using the wild-type peptide, GP2, or the altered peptide, I2L, pulsed onto DCs, C1R-A2 transfectants, or an $A2^+$ EBV-transformed B cell line. For these experiments, DCs, human C1R-A2 transfectants, or human $HLA\text{-}A2^+$ EBV-LCL cells pulsed with either the wild-type peptide, GP2, or the altered peptide, I2L, were adoptively transferred i.v. I2L was chosen, as this altered peptide showed the greatest ability to stabilize MHC complexes in vitro. Mice were sacrificed after the fourth cell transfer, splenocytes were isolated, and CTL assays were performed. We were unable to detect T cell responses from the spleen after i.v. administration of DCs pulsed with the wild-type peptide GP2 or after stimulation with either GP2 or I2L pulsed onto EBV-LCLs or C1R-A2 cells (Fig. 3). In contrast to GP2, I2L-pulsed DCs were able to induce significant CTL activity after four in vivo vaccinations (Fig. 3). Similarly, we did not find CTL responses in $A2K^b$ mice after administration of GP2-pulsed DCs injected either i.d. or s.c. (data not shown). Therefore, DCs pulsed with GP2 are unable

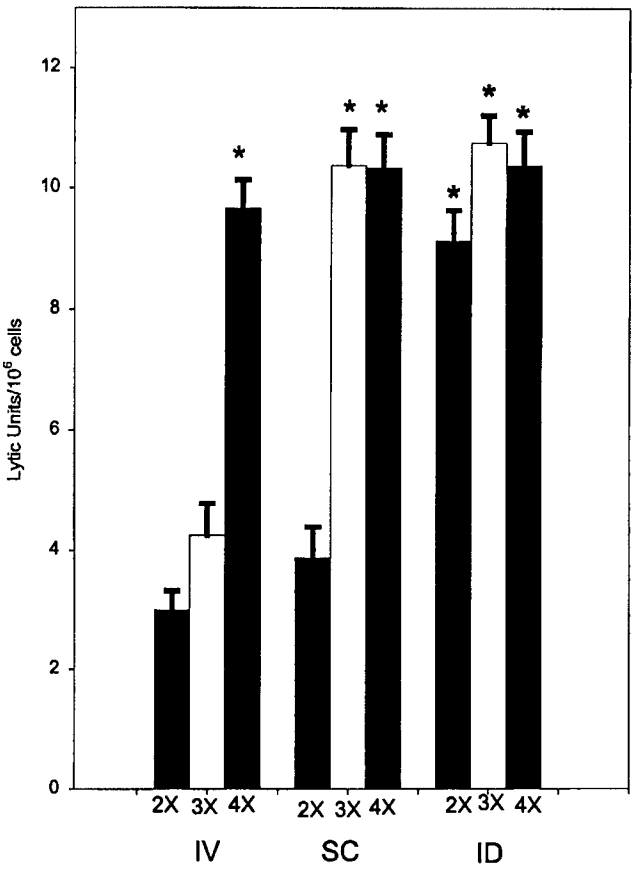

FIGURE 5. CTL responses after administration of peptide-pulsed DCs i.v., s.c., or i.d. A2-K^b mice were vaccinated s.c., i.d., or i.v. with 5×10^5 I2L-pulsed DCs every 7–10 days. Mice were sacrificed after two, three, and four vaccinations, and the draining lymph nodes (for s.c. or i.d. vaccinations) or spleen (for i.v.) were removed. Lymphocytes were isolated and tested for activity against C1R-A2 transfectants incubated with the wild-type peptide or against C1R-B7 transfectants incubated with either the wild-type peptide or an irrelevant $H2K^d$ binding peptide from *Listeria monocytogenes*. No lytic activity was found using C1R-B7 transfectants incubated with either the wild-type or irrelevant peptide. The data are pooled from two separate experiments, which included five mice in each group. Spontaneous release from all target cells was <10%. The p values were determined using Student's t test and comparing CTL activity using the second s.c. injection as the control. *, $p < 0.001$.

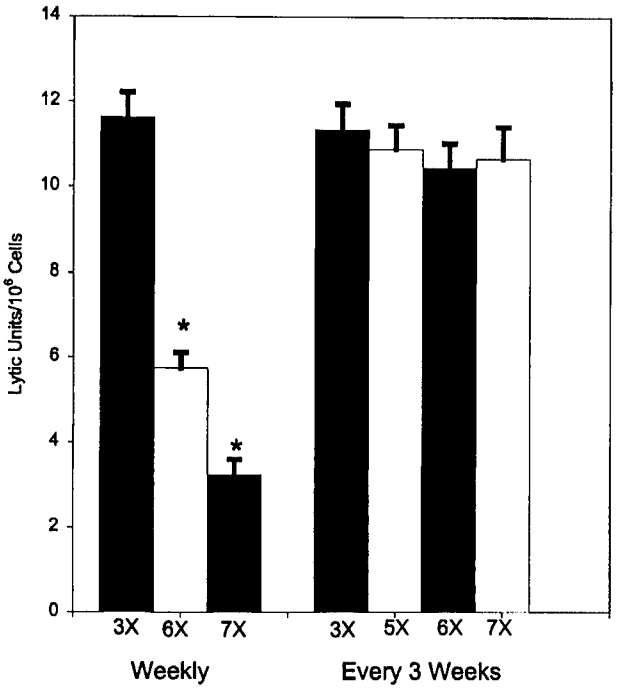

FIGURE 6. CTL activity does not diminish in mice vaccinated every 3 weeks with peptide-pulsed DCs. Groups of four $A2K^b$ were vaccinated either every 7–8 days or every 21 days with I2L-pulsed DCs (5×10^5) s.c. Mice were sacrificed after three to seven vaccinations, and lymphocytes were isolated from the draining lymph nodes and tested for CTL activity in a conventional ^{51}Cr release assay using C1R-A2 and C1R-B7 target cells (5000/well). Target cells were pulsed with either the wild-type peptide or an irrelevant peptide that binds to $H2K^d$. The data are pooled from two separate experiments. *, $p < 0.001$.

to induce CTL responses in $A2K^b$ mice, and this lack of response is not route dependent. Furthermore, the lack of a CTL response observed after the administration of I2L-pulsed C1R-A2 transfectants or $A2^+$ EBV-LCL suggests that the CTL response is not due to cross-priming and presentation of the epitope by mouse APCs.

We next evaluated whether CTL responses using APL-pulsed DCs correlated with the binding affinity of the APL for HLA-A2. DCs were pulsed with either I2L or L9V, which has a poorer binding affinity for HLA-A2 than I2L. Mice were given four i.v. vaccinations and then sacrificed (Fig. 4). Interestingly, there was an increase in CTL activity in animals that received DCs pulsed with the poorer binding epitope L9V compared with that in animals pulsed with I2L. Thus, while the initial CTL response is strongly influenced by the stability of the peptide MHC complex, the small degree of enhanced stability of HLA-A2 found using I2L compared with L9V did not augment the CTL response in vivo.

Route of administration in determining T cell responses

The CTL response after DC vaccination differs depending on the route of administration (35). There are conflicting data as to whether the efficacy of DC-based vaccines differs depending on the route used. To investigate this, $A2K^b$ mice were injected with I2L-pulsed DCs given i.v., s.c. (in the flank), or i.d. (footpad). After injection of peptide-pulsed DCs, we evaluated whether the anatomic site of activity and the timing and magnitude of the CTL response differed. Significant CTL activity was detected in cultures prepared from lymphocytes isolated from the draining lymph nodes after two i.d. injections and three s.c. injections. We did not detect significant CTL activity from mesenteric lymph nodes or lymph nodes isolated from the base of the tail after four i.v. injections. CTL activity was found in cultures prepared from lymphocytes isolated from the spleen after four i.v. injections (see Fig. 5). Thus, peptide-pulsed DCs given i.d. resulted in the most rapid CTL response, which occurred in the local draining lymph nodes. Adoptive transfer of DCs given i.v. induced measurable CTL responses only in the spleen. However, while the time to onset of the CTL response was different, the magnitude of the response did not differ depending on the route of administration. Regardless of the route of administration, CTL activity was induced after peptide-pulsed DC administration.

Repeated DC injections cause diminished CTL activity

For DC-based immunotherapy in patients (5, 10, 14, 15), multiple vaccinations are administered to generate CTL responses. To investigate whether the injection schedule impacted on the timing and magnitude of a CTL response, peptide-pulsed DCs were injected s.c. into $A2K^b$ mice either weekly or every 3 wk for seven treatments. CTL responses were determined from the draining lymph nodes after the third to seventh vaccination. We found that

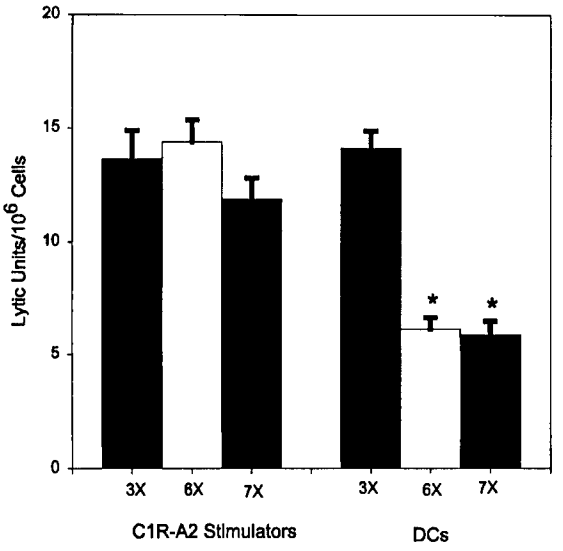

FIGURE 7. CTL activity does not diminish after vaccination with peptide-pulsed C1R-A2 cells. Groups of four $A2K^b$ mice were injected s.c. with 5×10^5 I2L-pulsed DCs every 7–8 days. After the third vaccination, half the mice received I2L-pulsed DCs every 7–8 days for four more treatments. The other half received I2L-pulsed C1R-A2 transfectants. Mice were sacrificed 24 h after the last vaccination, and lymphocytes were isolated from the draining lymph nodes. CTL assays were performed using 5000 EBV-LCL target cells that were HLA $A2^+$ incubated with either the wild-type peptide or an irrelevant peptide that binds to $H2K^d$. Control wells contained EBV-LCL that were HLA A2 negative with the irrelevant or wild-type peptide. Spontaneous release from target cells was <10% in all conditions. The data are pooled from two separate experiments, with four mice per group. *, $p < 0.001$.

CTL responses peaked after the third injection, but then, unexpectedly, diminished after the sixth and seventh injections in mice that were administered I2L-pulsed DCs weekly (Fig. 6). This reduction in CTL activity was not observed in mice that received DC vaccinations every 3 wk.

A conceivable hypothesis regarding the decrease in CTL activity is that the peptide-specific CTL had migrated from the draining lymph nodes into the systemic circulation after the sixth vaccination. To test this, we isolated splenocytes from mice given weekly s.c. injections and tested these lymphocytes 24 and 48 h after the sixth vaccination for the ability to lyse target cells pulsed with the wild-type peptide. No CTL activity was found from splenocytes isolated on either day after the sixth s.c. injection (data not shown).

Another possible explanation for the diminished CTL activity found after the administration of peptide-pulsed DCs weekly is enhanced apoptosis of activated CTLs by DCs. To evaluate this, we determined whether the administration of an APC that could not induce CTL responses, such as peptide-pulsed C1R-A2 cells, led to a similar reduction in CTL activity. All mice received I2L-pulsed DCs s.c. weekly for three vaccinations. After this initial priming, half the mice received the same treatment for three more injections. The other half of the cohort received s.c. injections with C1R-A2 cells pulsed with the I2L peptide (Fig. 7). Again, after vaccination with I2L-pulsed DCs given weekly, we observed a decrease in the CTL response after the sixth injection. By comparison, mice that received three weekly injections of peptide-pulsed DCs followed by weekly injections with C1R-A2 transfectants maintained CTL responses over the entire protocol.

Discussion

Vaccination of patients with specific malignancies, such as metastatic melanoma or breast or prostate cancer, with DCs charged with tumor peptides or tumor lysates or transfected with tumor RNA or DNA has rapidly gained interest in the tumor immunology community. There are currently several significant problems that need to be addressed for progress in this area. Many of the epitopes derived from tumors are from self proteins and as a result are poor immunogens. In addition, the mode of delivery of autologous DCs may promote poor CTL responses or, perhaps, tolerance. Finally, as CTL responses are infrequently generated after one vaccination, multiple treatments are needed to elicit a response. The timing of vaccination may be critical to induce CTL responses. Here, using the poorly immunogenic epitope from HER-2/Neu GP2, we have investigated these specific problems. We show that altering a peptide to enhance the interaction of the peptide with HLA-A2 is not effective in inducing a CTL response to the wild-type peptide unless the APL is presented by DCs. Furthermore, the intensity of the CTL response after autologous DC vaccination is not dependent on the route of administration of peptide-pulsed DCs, although the site of activity and timing are. Finally, we show that administration of DCs on a weekly schedule caused a significant decrease in CTL activity after the sixth vaccination. This was not found using either an every 3-wk vaccination schedule or an inferior APC such as C1R-A2 cells.

Previous investigators have shown that the dissociation rate of a peptide for the class I heavy chain complex is a strong determinant of the immunogenicity of that peptide (20, 33, 36). Of the 50 peptides we have evaluated that bind to class I molecules, the GP2 peptide has the poorest measurable binding profile (E. J. Collins, unpublished observations). Thus, this is a natural candidate epitope to alter in an attempt to improve its immunogenicity. We have found that there may be a threshold dissociation rate that is critical in the initiation of T cell responses using peptide-pulsed DCs. We were unable to generate responses regardless of the mode of delivery of the peptide using the wild-type peptide, which has a half-life of 24 min. Above this threshold, we found that a small enhancement of binding, as shown using the I2L peptide (half-life, 1.8 h) compared with L9V (half-life, 42 min), did not improve CTL responses after peptide-pulsed DC vaccination.

It is interesting that we and others have been unable to generate responses to the GP2 peptide in HLA-$A2K^b$ transgenic mice. The addition of the K^b $\alpha 3$ domain should have no effect on peptide binding. Lustgarten et al. (37), using mice transgenic for both human CD8 and HLA-A2, were able to generate responses to several different epitopes from HER-2/Neu, but not to the GP2 peptide. Similarly, we have been unable to generate responses to the GP2 peptide after treating mice with peptide in IFA, peptide alone (J. S. Serody, E. J. Collins, and J. A. Frelinger, unpublished observations) or peptide-pulsed DCs. Previously, investigators have shown that A2 transgenic mice have a similar T cell repertoire as humans (38). The absence of a response in these mice to the GP2 peptide suggests differences in the fine specificity of T lymphocytes between species. Alternatively, responses to GP2 from patients could be due to stimulation previously by cross-reacting epitopes.

Previous work in vivo in mice and in humans has suggested that the mode of delivery of DCs is important in eliciting T cell responses (35) (39). DCs given i.v. localized in the reticuloendothelial system and lung, while those given locally homed to the lymphatic system. We found a similar result in the $A2K^b$ mouse. CTL responses were present from the draining lymph nodes after i.d. or s.c. injection. Intravenous injection resulted in responses in the spleen, with little activity in the mesenteric lymph nodes. The route of administration is important in the efficiency of stimulating CTL. The i.d. route was the most effective method, which may be due to the local inflammatory response that occurred after footpad injection. This would result in an increase in the concentrations of

both cytokines and chemokines that are critical to migration (40–45) of DCs from the site and maturation (46, 47). However, we did not find that the route of administration was important in whether a response occurred or the intensity of this response. Additionally, our data suggest that the specific location of tumor cells may be important in the mode of delivery of DCs pulsed with peptides. The i.v. route may be preferable for vaccine administration against leukemia targets, while the i.d. or s.c. route may be preferable for responses to tumors localized in tissues.

As many current vaccine protocols use multiple vaccinations, we were interested in determining whether the schedule of these vaccinations affected CTL activity. Interestingly, we found that weekly administration of DCs s.c. resulted in diminished CTL activity after six treatments. The reasons for the decrease in CTL activity after weekly injection of peptide-pulsed DCs are not entirely clear. We were unable to show CTL activity in the spleen after the sixth s.c. administration of peptide-pulsed DCs, which suggests that the decrease in response is not due to redistribution of the CTL into the systemic circulation.

One possibility may be that weekly administration of peptide-pulsed DCs leads to clonal exhaustion of the responding T cell population. Because DCs are so effective at stimulating T cell responses, the significant T cell expansion associated with the use of DCs for vaccination may result in enhanced activation-induced cell death of the responder population. Using an APC such as C1R-A2 cells, which were unable to induce an initial CTL response, CTL activity was not diminished. This result may be due to decreased CTL activation induced by C1R-A2 cells, which would leave a residual population of peptide-specific T cells after each injection. This hypothesis can be evaluated currently using MHC tetramers to follow the responding population of T cells in vivo. If diminished CTL activity is found after multiple vaccinations using other peptides pulsed onto DCs or DCs transfected with tumor DNA or RNA, clinical studies may need to be modified to include either fewer treatments or the use of less stimulatory APCs. This also suggests that tolerance induced by DCs may be a function of the number of vaccinations of peptide-pulsed DCs and not of the route. If this proves to be true, frequent administration of peptide-pulsed DCs may provide a strategy to treat T cell-mediated autoimmunity.

Our data suggest that the presentation of APLs by autologous DCs can initiate a T cell response against a poorly binding self-epitope. These data also suggest that the use of in vitro models of peptide affinity may be helpful in the design of tumor vaccines. Using the I2L peptide pulsed onto autologous DCs, we have generated CTL responses in three naive individuals that are capable of lysing $A2^+$ ovarian cancer cell lines from two different individuals that overexpress HER-2/Neu (J. S. Serody, unpublished observations). These CTL were not able to lyse an $A2^+$ EBV-transformed B cell line from the patients with ovarian cancer, suggesting that a large number of peptide MHC complexes need to be present on the cell for lytic activity. Thus, self-peptides that bind poorly to MHC molecules are unlikely candidates to induce autoimmunity. Whether this approach results in enhanced killing of tumor cells will require characterization of in vivo CTL activity after clinical vaccination with APL-pulsed DCs. We are currently performing this evaluation.

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